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Anabolic-Androgenic Activities of Some Dimethylhydrazone Derivatives of Testosterone.* (26912)

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Undesirable masculinization has long been a liability to the use of testosterone and its esters as anabolic agents. The introduction of 17alpha-ethyl-19-nortestosterone(1) marked the initial step in separation of the androgenic from the anabolic component, while retaining the latter activity. In their recent review of the anabolic steroids Berczeller and Kupperman(2) included in their listing of the clinical problems in which these drugs are indicated such situations as the agonadal state, osteoporosis, postoperative catabolism, chronic administration of corticosteroids, retarded growth in pediatrics, and aging in geriatrics. The authors point out further that, in spite of the numerous clinical indications for anabolic agents, "a universally acceptable anabolic agent without untoward side effects has yet to be developed."

Clinton *et al.*(3) reported synthesis of several steroidal [3,2-c] pyrazoles with varied endocrinologic activities. Arnold *et al.*(4) and Potts *et al.*(5) have described the anabolic-androgenic activities of andro-stanazole (17beta-hydroxy-17alpha-methylandrostano-[3,2-c]pyrazole). In light of the high order of activity of this compound it was of interest to examine the testosterone-like activity of certain dimethyl-hydrazone derivatives of testosterone.

Methods. The compounds (Table I) were administered subcutaneously as solutions in sesame oil in a volume of 0.1 cc per day. Anabolic-androgenic activities were determined in castrated, 21-day-old male rats of the Mead Johnson colony (McCollum strain) by noting the increases in weight of the levator ani muscle, seminal vesicle, and ventral prostate gland, as described by Hershberger *et al.*(6). The relative anabolic-androgenic activities were calculated as follows: L.A./V.P. ratio = (levator ani response of test group—L.A. response of control)—(ventral prostate response of test group—V.P. response of control) (6).

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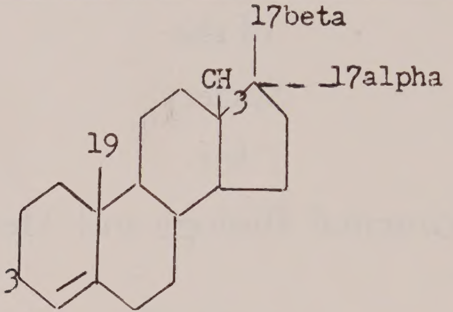
* The compounds described herein were synthesized by Dr. R. H. Wiley, Dept. of Chemistry, Univ. of Louisville, Ky.

TABLE I. Structural Relations of Certain Dimethylhydrazone Derivatives of Testosterone.



Compound name	Compound No.	3	17	19
testosterone dimethylhydrazone	I	$(CH_3)_2N{-}N{=}$	–OH beta	CH_3
methyltestosterone dimethylhydrazone	II	$(CH_3)_2N{-}N{=}$	–CH_3 alpha; –OH beta	CH_3
19-nortestosterone dimethylhydrazone	III	$(CH_3)_2N{-}N{=}$	–OH beta	H
cis testosterone dimethylhydrazone	IV	$(CH_3)_2N{-}N{=}$	–OH alpha	CH_3
testosterone propionate	V	=O	–O–C:O–C_2H_5 beta	CH_3
norethandrolone (Nilevar®)	VI	=O	–C_2H_5 alpha; –OH beta	H

Estrogenic activity was assayed by the method of Lauson *et al.*(7), measuring the increase in uterine weight in 21-23-day-old, female rats. Progestational activity was studied by the deciduoma test in adult, ovariectomized rats as outlined by Zarrow *et al.* (8), using an intraluminal injection of 1.0 mg histamine dihydrochloride in the right horn as the pseudofertilization stimulus.

Results. Of these 4 dimethylhydrazone derivatives of testosterone only compound II exhibited significant androgenic activity on both the seminal vesicle and ventral prostate gland (Table II). Compound III showed relative, but insignificant, anabolic activity. On an absolute basis compound III was not even of the order of potency of testosterone propionate (V) and was considerably weaker than the highly active anabolic agent, norethandrolone (VI; Nilevar®). Undoubtedly as a reflection of the fact that it is a 19-nortestosterone derivative, the data obtained with compound III did reveal a relatively greater anabolic component than in compounds I, II, IV, or V. In this regard the rats receiving compound III actually showed a slight decrease in the response of the seminal vesicle. The only other compound, including norethandrolone (VI), with which this was evident was compound IV, whose overall activity was in fact negligible. As with compound VI, compound III was less active orally than parenterally.

Similar dimethylhydrazone derivatives of pregnenolone, progesterone, and ethisterone showed no progestational activity in the deciduoma test in estrone-primed, ovariectomized rats, and estrone dimethylhydrazone was only 1/32nd as active as estrone itself in increasing the uterine weight of immature rats.

Discussion. This relatively small series of dimethylhydrazone derivatives of the 3 principal types of sex hormones can not be construed as sufficiently large to draw extensive conclusions regarding structure-activity relationships. However, the relatively negative findings are sufficiently clear-cut to indicate that substitution of the dimethylhydrazone moiety tends to reduce markedly the

TABLE II. Anabolic-Androgenic Activities of Certain Dimethylhydrazone and Other Steroids.

Compound No.	Dose, μg/rat s.c.[*]	No. rats	Anabolic response (levator ani)	Androgenic response (ventral prostate)	Androgenic response (seminal vesicle)	L.A./V.P. ratio[‡]
			% final body wt × 1000[†]			
I	0	10	25.6 ± 1.5[‖]	6.8 ± .4[‖]	6.6 ± .6[‖]	—
	25	9	25.8 ± 1.1	9.1 ± .3	5.6 ± 1.4	.09
	200	9	30.2 ± 1.7	25.3 ± 2.3	10.2 ± .8	.25
II	0	9	30.1 ± 1.7	11.9 ± 1.3	8.3 ± .4	—
	25	10	32.3 ± 1.7	24.9 ± 3.2	13.4 ± 1.7	.17
	200	10	34.2 ± 2.4	64.7 ± 8.9	39.6 ± 4.6	.08
III	0	8	31.8 ± 2.0	10.6 ± .5	10.5 ± 1.0	—
	25	9	31.6 ± 1.4	11.3 ± .7	9.7 ± .8	−.3
	200	10	35.6 ± 2.9	15.6 ± 1.8	8.1 ± .8	.76
III	0	8	31.7 ± 1.0	11.0 ± .6	9.1 ± .5	—
p.o.[§]	100	10	34.4 ± 1.9	15.0 ± .9	9.8 ± .4	.68
	400	10	35.5 ± 1.6	17.6 ± 1.5	9.8 ± .8	.58
IV	0	8	26.7 ± 1.3	11.2 ± .4	8.6 ± 1.2	—
	25	10	27.0 ± 1.7	9.9 ± .5	8.8 ± .8	−.23
	200	10	26.8 ± 1.0	17.4 ± 1.4	7.5 ± .4	.02
V	0	52	27.0 ± .6	10.4 ± .5	8.5 ± .3	—
	12.5	17	32.5 ± 1.4	38.0 ± 1.9	24.3 ± 1.3	.17
	100	22	52.8 ± 1.8	93.9 ± 2.9	86.1 ± 2.6	.3
VI	0	9	27 ± 1.9	10.8 ± 1.3	8.1 ± .4	—
	12.5	10	52.2 ± 7.8	13.5 ± 1.1	8.5 ± 1.0	9.3
VI	0	10	28.5 ± 1.8	9.5 ± .6	7.9 ± .4	—
p.o.[§]	25	9	31.0 ± 1.2	10.6 ± .7	9.3 ± 1.0	2.3
	200	10	39.2 ± 1.7	12.5 ± .9	7.2 ± 1.0	3.6

[*] Animals dosed s.c. immediately post-castration and for 6 days thereafter, and sacrificed 24 hr following 7th dose; dosage vol 0.1 cc; controls received 0.1 cc vehicle (sesame oil) only.

[†] % final body wt × 1000 = (tissue wt in g ÷ final body wt in g) × 100000.

[‡] L.A./V.P. ratio calculated as described by Hershberger *et al.* (text ref. No. 6) to obtain index of anabolic activity: (levator ani response of test − L.A. response of control) ÷ (ventral prostate response of test − V.P. response of control).

[§] Note oral route; methocel vehicle; ingestion vol 0.25 cc.

[‖] Mean ± S.E.

inherent activity of the parent compound. In the case of compound III, the hydrazone substituent did not block completely the qualitative separation of anabolic-androgenic activities that is characteristic of 19-nortestosterones.

That some arrangement of the –N–N= grouping on the steroid is not absolutely deleterious to the molecule is evident when one compares the data reported by Potts *et al.* (5) for andro-stanazole with that described herein for compound II, the compound in this series structurally most comparable to the cyclized andro-stanazole. The two sets of data indicate that andro-stanazole is infinitely more anabolic than compound II, but the latter is 1.5 to 2.5 times more androgenic. Exactly how the cyclic nature of the =CH–NH–N= system in andro-stanazole favors anabolic activity, especially upon oral administration, is uncertain, but the data reported herein indicate that the open-chained hydrazone system is more likely to cause a reduction in activity.

Summary. 1. Eight dimethylhydrazone derivatives of the sex hormones have been examined for anabolic, androgenic, estrogenic, and progestational activities in standard assay procedures in rats. 2. The dimethylhydrazone substituent induced a significant reduction in absolute activity relative to the parent compound, but it did not obliterate the qualitative separation of anabolic-androgenic activities in the 19-nortestosterone derivative.

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Identification of Heterozygous Carriers of Gargoylism. (26913)

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In recent years gargoylism (Hurler's syndrome, dysostosis multiplex) has been found to be a hereditary disorder of acid mucopolysaccharide (AMPS) metabolism(1-3). Thorough evaluation of pedigrees of affected families has indicated 2 forms of gargoylism: the autosomal recessive and the sex-linked recessive types(4). Many patients with overt gargoylism can be identified by an elevated urinary level of total AMPS(2-4) and by the presence of abnormal acid mucopolysaccharides such as chondroitin sulfate B(2-4) (ChS-B) and heparin monosulfate(3,5) (HMS) in their urine. Up to the present time, however, it has not been possible to identify chemically the heterozygous carriers of the gargoylism disease trait.

For the characterization of acid mucopolysaccharides, Hoffman and co-workers(6) have used the hexuronic acid carbazole/orcinol (C/O) color extinction ratio. In an analogous manner we have used the carbazole (7)/naphthoresorcinol(8) (C/N) color ratio. We obtained a C/N color ratio of 13.6 for chondroitin sulfate A* (ChS-A), 0.6 for chondroitin sulfate B*, 14.0 for chondroitin sulfate C* (ChS-C), and 12.2 for heparin monosulfate†. The C/O color ratios reported by Hoffman and co-workers(6) for ChS-A and ChS-C are only about 4 times the C/O ratio for ChS-B whereas the C/N color ratios we obtained for ChS-A and ChS-C are about 20 times the C/N ratio for ChS-B. A low C/N ratio appears to be characteristic of ChS-B.

The usefulness of the AMPS C/N ratio was discovered in our search for better methods of detecting the ChS-B and the elevated level of total AMPS in the urine of patients with clinical gargoylism. In some cases a simple quantitative index of the AMPS in urine was found to be inadequate for chemical verification of an abnormal AMPS excretion. Addition of the C/N ratio as an index of urine AMPS quality to the index of AMPS quantity has proved to be much more specific than the quantitative index alone. Our results indicate that patients with suspected gargoylism as well as their normal parents and other relatives with the heterozygous trait of gargoylism can now be identified.

Our method, performed on a single urine specimen, involves preliminary dialysis to remove most of the smaller uronic acid-containing molecules; concentration of the AMPS by precipitation with 5% cetyltrimethyl-ammonium bromide according to the DiFerrante and Rich(9) procedure; and determination of the AMPS hexuronic acid by the carbazole method of Dische(7) and the naphthoresorcinol method of Pelzer and Staib(8). The quantity and quality of the AMPS is then represented respectively as a

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* We gratefully acknowledge the generous gift of purified chondroitin sulfate A, B, and C from Dr. K. Meyer, New York.

† We wish to thank Dr. J. A. Cifonelli, Chicago, for supplying purified heparin monosulfate.

TABLE I. Urinary Excretion of Acid Mucopolysaccharides by Normal Persons, Patients with Gargoylism, and Heterozygous Carriers.

		Mean ± S.E.*	S.D.†	P‡
Normal persons (all ages)	C/Cr§	5.1 ± .7	4.1	
	C/N‖	15.1 ± .9	5.6	
Patients	C/Cr	53.8 ± 7.1	25.7	<.01
	C/N	4.2 ± .3	1.2	<.01
Suspected heterozygous carriers	C/Cr	3.6 ± .6	3.1	>.1
	C/N	4.5 ± .3	1.5	<.01

* Mean value ± stand. error of mean.

† Stand. dev.

‡ P value for comparison with normal excretion.

§ $\text{C/Cr ratio} = \frac{\text{mg of carbazole-hexuronic acid}}{\text{g creatinine}}$

‖ $\text{C/N ratio} = \frac{\text{mg of carbazole-hexuronic acid}}{\text{mg of naphthoresorcinol-hexuronic acid}}$.

carbazole/creatinine (C/Cr) ratio

$$\left(\frac{\text{mg of carbazole-hexuronic acid}}{\text{g of creatinine before dialysis}}\right)$$

and a carbazole/naphthoresorcinol (C/N) ratio

$$\left(\frac{\text{mg of carbazole-hexuronic acid}}{\text{mg of naphthoresorcinol-hexuronic acid}}\right).$$

The mean C/Cr ratio and C/N ratio of the AMPS excreted by 38 normal human subjects of both sexes and all ages, by 13 patients with gargoylism, and by 27 suspected heterozygous carriers of gargoylism are compiled in Table I. Both the mean C/Cr ratio and the C/N ratio of the patients were significantly different from normal values, the C/Cr ratio being much higher and the C/N ratio lower than normal. The suspected heterozygous carriers revealed a normal mean C/Cr ratio but a C/N ratio which was significantly lower than the normal mean C/N ratio and of the same order as the C/N ratios in patients with overt manifestations of gargoylism. The low C/N ratios suggest that a certain proportion of the assayed AMPS material consists of ChS-B. Since ChS-B is not normally excreted in the urine(2-4), the occurrence of low C/N ratios in some clinically healthy relatives of patients with gargoylism most likely indicates the heterozygous carrier state of the disease trait. Actual isolation and identification, however, of ChS-B from these urines has not been performed.

C/N ratios obtained from 10 patients with gargoylism and their respective parents are shown in Table II. In 7 instances both father and mother revealed low C/N ratios, suggesting an autosomal recessive mode of inheritance. In one family the father had a normal C/N ratio which would indicate the sex-linked recessive type of transmission.

We did not find significant difference in the nature of AMPS excreted by patients with the autosomal recessive and the sex-linked recessive form of gargoylism. Also, we have not had an opportunity to examine the urinary AMPS of a patient who excreted only HMS as described by Harris(5).

Summary. The carbazole and naphthoresorcinol methods for hexuronic acid deter-

TABLE II. Urinary Acid Mucopolysaccharides Excreted by Patients with Gargoylism and Their Parents.

		C/N ratios*			
Case	Sex	Patient	Father	Mother	Suggested mode of inheritance
1	♂	6.0	10.0	4.1	Sex-linked recessive
2	♀	3.3	5.0	2.3	Autosomal recessive
3	♀	3.6			
4	♂	2.6	3.3	4.0	” ”
5	♂	4.2	3.9	4.0	” ”
6	♂	5.1	3.4	4.7	” ”
7	♂	5.0	4.5	2.7	” ”
8	♂	3.7	7.5	5.9	” ”
9	♂	4.8			
10	♂	5.0	6.4	5.9	” ”

* $\text{C/N ratio} = \frac{\text{mg of carbazole-hexuronic acid}}{\text{mg of naphthoresorcinol-hexuronic acid}}$ (normal range: 15.1 ± 5.6).

mination have been used to characterize the partially purified acid mucopolysaccharides obtained from urine of normal persons, from patients with gargoylism and from their relatives. The results, expressed as a ratio of carbazole to naphthoresorcinol hexuronic acid, demonstrated a similar abnormal qualitative excretion of chondroitin sulfate B by patients with gargoylism, by one or both parents, and by some of their other relatives. Occurrence of an abnormal qualitative excretion of chondroitin sulfate B in the clinically normal relatives of patients with gargoylism probably indicates the heterozygous carrier state of the disease.

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Rate of Fat Uptake by Intestinal Lymphatics.* (26914)

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The time course of fat absorption has been studied most frequently by following the changes in blood lipid concentration at intervals after fat meals using either the chylomicrograph(1,2) or chemical methods(3) for analysis of plasma lipids. Disappearance of the excess lipid from the circulation after fat feeding has often been taken as an index of the completion of fat absorption, but this is based upon at least 2 important assumptions: 1) that the methods of measurement are sufficiently sensitive to detect extremely small amounts of absorbed lipid and 2) that the rate at which fat is added to the circulation during fat absorption always exceeds the rate at which it is removed. The time at which the excess lipid disappears following a fat meal is highly variable, ranging from 4 hours (1) to 10 hours(2) using either of the above methods. However, recent studies using the more sensitive I^{131}-labelled triolein technic have demonstrated the continued presence of I^{131}-labelled lipid in the circulation for periods extending beyond 24 hours after its oral administration(4) suggesting either a delayed removal rate or a prolonged absorption period. In the present study evidence will be presented to show that absorbed fat may continue to enter the circulation through the thoracic duct for 24 hours or more after a single oral fat load. Some of the reasons for this prolonged absorption period are examined and discussed in relation to the above mentioned studies involving the simple measurement of plasma lipid concentration during alimentary lipemia.

Methods. The study was carried out in dogs ranging in size from 10 to 18 kg, maintained on a nutritionally adequate "fat-free" diet for a variable period of time prior to experiment. This diet contained: purified casein, 24%; sucrose, 40%; corn starch, 16%; cellulose, 14%; salt mixture (U.S.P. IV), 4%; complete vitamin mixture, 2%;

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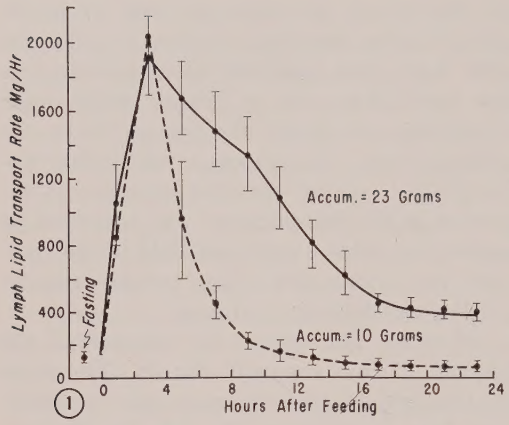
and was fed at the rate of 20 g/kg body weight/day. A fat-free diet was chosen because it was a later purpose to add known amounts of fat to this diet and to measure the rate at which the added fat was transported from the intestine through the lymphatic channels. The technic for lymphatic cannulation has been described(5). This operation was performed on the fasted animal under nembutal anesthesia, and the lymph was drained continuously into a flask which the animal carried. In 4 of the 15 dogs used, an indwelling lucite cannula was inserted into the duodenum to permit administration of the fat directly into the upper intestine. On the morning following the operation when the animals had fully recovered from the anesthetic, 25 g of olive oil were administered either by stomach or by duodenum. In the gastric feeding experiments, olive oil was either mixed and fed with the fat-free diet or administered alone by stomach tube. In the duodenal feeding experiments, olive oil was administered through the indwelling tube by syringe over a 5- to 10-minute period. In one case the animal was allowed to eat the fat-free diet immediately afterward. In all of the experiments lymph was collected at regular timed intervals (usually 2 hours) both prior to and following administration of the lipid. Post-feeding collections were continued for 24 hours. All lymph samples were measured for volume and aliquots were taken for total lipid measurement using the chloroform-methanol extraction procedure of Sperry and Brand(6).

In 2 separate experiments an effort was made to estimate the extent to which lipids absorbed through channels other than the thoracic duct might contribute to the lipids recovered from the lymph cannula. This was done because of the possible error inherent in the assumption that all of the excess lipid returned in the lymph after fat feeding is the result of a direct and unidirectional contribution from the intestine. Therefore, to test this assumption 2 thoracic duct fistula dogs were fed 50 g of olive oil and the lymph was collected until absorption was complete as evidenced by the visible clearing of the lymph on the following day. Three hundred to 400 ml of the milky citrated chyle obtained during the peak of the absorption period were then reinfused intravenously into the same dogs over a 2-hour period while continuing to drain the lymph from the thoracic duct. The extent to which the reinfused lipid escaped the vascular tree and appeared in the thoracic duct was estimated by measuring volume and total lipid in the thoracic duct lymph over 2-hour periods prior to, during and following infusion.

Results. The rate of fat transport in the thoracic duct is shown in Fig. 1. The mean fasting lymph lipid transport rate ± standard error is shown on the left for all dogs. This averaged 124 mg/hr after 24-48 hours of fasting and was independent of duration of the post-absorptive period. Attention is directed to the solid line representing the rate of lymph lipid transport in 11 dogs after receiving 25 g of olive oil by stomach. No apparent differences were noted between dogs receiving olive oil alone and those receiving olive oil mixed with the fat-free diet. Therefore, results from all stomach-fed dogs were pooled. Lymph lipid transport rate began to increase usually within the first hour after feeding and reached a peak whose average fell in the 2-4 hour interval. The time at which the peak occurred, however, was extremely variable, ranging from 3 to 9 hours. The maximum lipid transport rates achieved at the peaks were also highly variable averaging 1945 mg/hr but with a range of 1300 to 4800 mg/hr. Some of the animals exhibited sharp, highly elevated peaks early after feeding while others exhibited peaks which were less distinct, of lower magnitude and relatively delayed in time. This variability may perhaps reflect a variable stomach emptying rate among the different dogs. One other notable feature which the stomach-fed animals exhibited was an extremely prolonged absorption period, as evidenced by the fact that even at the end of 24 hours lipid transport rate was still significantly elevated above the average level found after 24-48 hours of fasting. In gross appearance the lymph still exhibited elevated turbidity 24 hours after a fat meal.

The dashed line of Fig. 1 shows mean rate



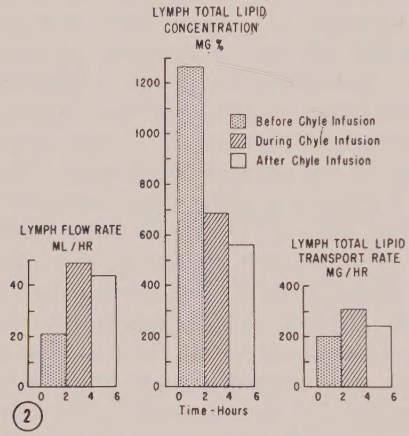


FIG. 1. Rate of fat transport by intestinal lymphatics. ●——● fed 25 g of olive oil by stomach at zero time. Mean of 11 dogs. ●– – –● fed 25 g of olive oil by duodenum. Mean of 4 dogs. Vertical bars represent stand. error. Accum. represents total 24 hr accumulated lipid transport.

FIG. 2. Flow rate and total lipid transport in thoracic duct lymph before, during and after intravenous infusion of 300-400 ml of homologous milky chyle. Mean of 2 dogs.

of lymph lipid transport in the dogs fed olive oil by duodenum. In this group the peak transport rate occurred in the 2-4-hour interval in all dogs. With respect to time at which the peak occurred and maximum rate of transport at the peak, the duodenum-fed dogs did not differ significantly from those fed by stomach. However, in the duodenum-fed group the transport rate had returned to the fasting level in approximately 13 hours. Statistical comparison of the stomach- and duodenum-fed groups from the time of the peaks to the end of the 24th hour revealed a significant difference at all intervals beyond the 7th hour ($P<0.01$). From these data it can be surmised that the prolonged absorption period in the orally fed dogs is in large measure the result of retention of fat in the stomach. The effectiveness of this retention factor in enhancing total absorption is revealed by the fact that the accumulated lymph fat output in the 24 hours was 23 g in the stomach-fed dogs and only 10 g in the duodenum-fed group.

That the recirculation of fat absorbed through other channels is not a significant factor in the delayed clearance is revealed by the experiments in which milky citrated chyle was reinfused intravenously into 2 thoracic duct fistula dogs in the post-absorptive state. In Fig. 2 it can be seen that the reinfusion process more than doubled the thoracic duct volume flow rate while almost halving total lymph lipid concentration. The total amount of fat transported increased slightly during the infusion period from 200 mg/hr to 309 mg/hr. Since the amount of lipid in the infused lymph averaged 19 g in the 2 dogs, this slight increase amounted to only 1.2% of the infused amount. This amount is probably negligible and serves to point out how effectively circulating lipids tend to be retained within the vascular tree or removed without any significant retransport in lymph.

Discussion. The results of the present study conflict with the widely held assumption that the process of fat absorption is completed within an interval of 10 hours or less following a single dose of fat by mouth. This assumption probably originated from experiments involving measurement of fat tolerance curves utilizing either chemical methods or the chylomicrograph to follow gross changes in blood total lipid concentration during absorption. It is unlikely, however, that these methods are sufficiently accurate to detect small changes in plasma lipids when the absorption rate is slow or when rate of removal of the lipid keeps pace with its rate of absorption. By measuring the lipid in the total volume of thoracic duct lymph prior to its entrance into the circulation one avoids the factor of utilization or removal and can conveniently and accurately measure both the time course of lipid absorption and total amounts absorbed. It must be assumed, however, that the thoracic duct constitutes the major pathway for transport of absorbed lipids and that any lipid entering the circulation through other channels will not escape the vascular tree and recirculate through the lymph cannula. The first of these assumptions has been tested in a number of laboratories(7,8,9) and it is now generally held that at least 80% of long chain dietary fats are absorbed from the intestine through lymphatic channels and transported in the thoracic duct. The second assumption was tested in the present study, and it was shown that only 1.0% of the lipid in intravenously injected homologous milky chyle could be accounted for in the thoracic duct lymph during a 2-hour infusion period. This finding agrees with that of French and Morris(10).

In the present study more than 24 hours were required for the lymph to clear completely following a single oral dose of olive oil. In view of the above considerations it seems reasonable to conclude that this delayed clearance is the result of a prolonged absorption period. A large part of this prolonged absorption period would appear to be due to retention of fat in the stomach because when the olive oil was administered directly into the upper intestine the lymph cleared in approximately 13 hours.

The results in the stomach-fed dogs are at variance with those of Simmonds(11) in the rat. This investigator found that all of the measurable excess fat output in lymph occurred in the first 10 hours after administering olive oil through a gastrostomy tube. However, since only 0.5 ml of olive oil was fed, it seems doubtful that the increases in the lymph lipids would be high enough to be detected throughout the entire absorption period. In the human, Malm *et al.*(4) found 2-4% of a dose of I^{131}-labelled olive oil still present in the circulation 24 hours after feeding 5-10 ml of the labelled fat by mouth. The present study supports this finding and provides suggestive evidence that the continued presence of absorbed fat in the circulation after 24 hours is the result, at least in part, of a continuing absorption from the intestine. Part of this prolonged absorption period is probably the result of the slow delivery of fat by the stomach into the upper intestine, but this slow delivery perhaps serves in the best interest of the organism by permitting a larger percentage of dietary fats to be absorbed. Other evidence in support of the importance of the stomach for dietary lipid assimilation is provided by the common clinical finding that profound losses of dietary fat in the stool may occur following gastrectomy (12).

Summary. Measurements of the rate of fat uptake by the intestinal lymphatics showed that when dogs were fed 25 g of olive oil by mouth the total lipids in the thoracic duct lymph remained elevated above fasting levels for more than 24 hours. When the olive oil was introduced directly into the duodenum, the lymph was cleared of excess lipid in 13 hours. That the excess lipid in postcibal lymph probably represents fat which has been directly contributed by absorption from the intestine was shown by the failure to observe a significant increase in thoracic duct lymph lipid content in response to infusing a large volume of milky chyle intravenously. These results suggest that absorption of fat continues for more than 24 hours after its oral administration and is associated with a slow and prolonged delivery of fat by the stomach into the upper intestine. Stomach-fed dogs returned a significantly higher percentage of the fed fat in the lymph in 24 hours than did duodenum-fed dogs suggesting that the retention of fat in the stomach enhances capacity for effective lipid absorption from the intestine.

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Sterol Synthesis by Cells Cultured on Serum from Heat-Stressed Chickens.* (26915)

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Stress has frequently been linked to increased serum lipid levels as an etiologic factor(1,2). Experiments with hypophysectomized and adrenalectomized animals have indirectly incriminated stress hormones in hyperlipemia(3). Adrenaline and adrenocortical hormones have been shown not only to produce hyperlipemia *in vivo*(4) but also *in vitro* in cultures of rat adipose tissue(5,6,7,8). Similarly, corticotropin has been found to increase unesterified fatty acid levels *in vivo* (9). These reports are largely concerned with stress-hormone-induced fatty acid mobilization, however, mirroring the present increased interest in the role of unesterified fatty acids (UFA) in over-all animal lipid metabolism.

Bailey, Gey and Gey(10) found that MBIII lymphoblasts *in vitro* remove cholesterol esters from the nutrient medium, presumably by pinocytosis, and expel unesterified cholesterol into the medium. Azarnoff (11) has observed that organ cultures of the coronary artery and aorta of chickens, guinea pigs and rabbits convert C^{14} acetate to cholesterol, whereas this is not a characteristic of humans, dogs, cats and rats, although the latter do synthesize some type of digitonin precipitable steroid. Simms *et al.*(12) obtained a so-called "B-factor" or "lipfanogen" from human and animal sera (including chicken), which evidently causes lipid aggregation, resembling spontaneous atherosclerosis, in organ cultures of chicken and human arteries. In none of these instances, however, have these phenomena been linked to stress.

Recently(13) studies were conducted by us on the use of tissue culture techniques in biological tests for ascertaining stress in animals. Mouse fibroblasts cultured on a medium containing "stressed" serum (hereafter referred to as S-serum) produce a large number of small lipid droplets. This phenomenon is not elicited by "non-stressed" serum (hereafter referred to as NS-serum). It is demonstrable in rapidly growing, healthy cultures and is not a manifestation of degeneration.

The present report consists of results of tests made in an attempt to determine the nature of the lipid found in these cells after exposure to serum from stressed animals.

Materials and methods. The "Low line" strain of mouse fibroblasts (NCTC #2445) (14) was used for these tests. They were cultivated by inoculating 300 to 500 thousand cells per ml into either T-15 or T-60 flasks containing 2 or 10 ml, respectively, of a 10% horse serum 109 medium. After growth was well established (average cell numbers 1.5 million cells per ml), this medium was replaced, in half of the cultures, with a balanced salt solution (B.S.S.) containing up to 20% serum from stressed chickens and in the other half with serum from non-stressed chickens. Young White Leghorn chickens (age: 3-4 months) were subjected to heat stress for 24 hours at 102°F as described in a previous report(13). Total cell numbers in both control and test cultures were essentially identical at the end of the test period

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TABLE I. Sterol Content of Mouse Fibroblasts (NCTC #2445) Cultured on Sera from Stressed and Non-stressed Chickens.

Conc. of serum in medium	Time of exposure to serum, days	Cholesterol content in mg/10^7 cells Non-stressed	Cholesterol content in mg/10^7 cells Stressed
12%	2*	.420	.571
20%	2†	.428	.555
10%	6*	.104	.150

* 3 exp. † 4 exp.

(approximately 0.9 million cells per ml at 48 hours; 3.4 million per ml at 6 days).

Sterol determinations were made by the Bloor-Sackett modification(15) of the original Liebermann-Burchard reaction on cell-free media before and after culturing at 37°C for 2 days, 3 days and 6 days respectively. Error in the procedure was found to be ± 1.63 mg % by multiple tests on known cholesterol standards. Cell extractions were prepared by washing all of the cells in a T-60 culture with B.S.S., after which they were extracted 3 times for 20 minutes each with 3:1 alcohol-ether solution. The combined extracts were evaporated to dryness over a water bath, the residue extracted 3 times with chloroform and this extract analyzed for sterol as above. Error in the procedure was found to be ± 0.22 mg %. It is recognized that many sterols give the same, or very nearly the same, color reaction as cholesterol with this technique(16). Other currently used methods(17), employing precipitation of the cholesterol digitonide in 96% alcohol, followed by colorometric analysis, are apparently more specific, but even they are open to question. Azarnoff(11) studying utilization of sodium-acetate-C^{14} by arterial tissue in organ culture, found that human aorta and coronary artery were capable of incorporating this substance into a digitonin-precipitable substance, but that this substance was *not* cholesterol, since "all of the radioactivity disappeared from the cholesterol fraction with preparation of the dibromide." Others have observed the same(18). Because of this lack of specificity, we prefer to use the term sterol concentration rather than cholesterol concentration, but feel that this terminology does not alter the significance of the findings.

Results and discussion. Tests were run to ascertain sterol content, and Table I presents the results with cell extractions. There is a definite increase in the amount of sterol in cells cultured for 48 hours on serum from stressed animals. Longer exposure (6 days) does not increase the amount. Although the total amount of sterol was decreased in cells cultured in S- and NS-serum at the end of 6 days, relative difference was of the same order as that in cells exposed for only 2 days. Concentrations of serum in the medium apparently had no significant effect on total sterol in the cells. The data suggest that sterol synthesis is stimulated by "stressed" serum but that the product is not retained within the cell.

Table II presents results of analyses of the media before and after exposure to the cells. The change in sterol content of the NS-serum component after 48 hours culture was negligible, varying from –0.67 to 1.19 mg% (average 0.37 mg %). In S-serum the sterol content increased considerably. The increase varied between 6.66 to 13.88 mg % (average 9.92 mg %). In another experiment, the cells were cultured for 6 days in 10 ml of medium containing 10% S- or NS-serum. Every 48 hours the medium was replaced by 10 ml fresh medium. Thus, at the end of the experiment, there were 30 ml of pooled medium to analyze in each instance. The sterol content of these specimens represented total

TABLE II. Sterol Content in Serum Component of Media in Which Mouse Fibroblasts (NCTC #2445) Have Been Cultivated.

Sample No.	Conc. of serum, %	Sterol conc. in mg % Unused medium	Sterol conc. in mg % Medium after 48 hr culture	Sterol conc. in mg % Increase
"Non-stressed" serum				
1	12	98.14	98.90	.76
2	20	88.25	88.84	.59
3	20	88.25	89.44	1.19
4	20	88.25	88.25	.00
5	20	88.25	87.58	–.67
"Stressed" serum				
1	12	113.89	127.77	13.88
2	12	118.05	127.77	9.72
3	20	125.33	136.00	10.67
4	20	136.00	142.66	6.66
5	20	125.33	133.33	8.00
6	20	125.33	136.00	10.67

TABLE III. Characteristics of Lipid Droplets Produced in Media Containing UFA or Serum from Stressed Chickens.

	Oleate induced	Stressed serum induced
Size:	Large, up to 15 μ	Small, about 1-2 μ
Rate of formation:	Appear in 6 hr, fully formed in 24 hr	Appear in about 12 hr, fully formed in 48 hr
Rate of utilization:	48 hr	Not utilized
Heat inactivation:	Not inactivated	Inactivated by 89°C for 30 min.
Insulin-glucose effect:	Inhibits formation	No effect

output of these cells over a 6-day period under nearly ideal growing conditions. Total sterol increase in NS-medium was found to be 5.79 mg %; in S-medium it was 26.40 mg % or 4½ times as much.

The above data show that sterol does accumulate in the medium in contrast to intracellular sterol, which does not accumulate (Table I). These observations suggest that intracellular synthesis of sterol is increased under S-serum stimulus, and that the product escapes into the surrounding medium where it accumulates. We have, in fact, observed large accumulations of extracellular lipid in chick embryo heart and artery explants grown in presence of S-serum. Control organ cultures grown on NS-serum medium did not show a similar demonstrable aggregation of lipid. Although past experience indicated the extra cellular cholesterol is not the source of these lipid droplets(13), another experiment was carried out to test this fact further. Cholesterol was added to normal horse serum in concentrations equivalent to an increase of 33 mg % and 67 mg %. The mouse fibroblast cells were grown on this medium for 48 hours without production of lipid droplets. The same experiment was repeated using medium with non-stressed chicken serum with the same results. The evidence here suggests that exogenous cholesterol plays little if any part in the appearance or non-appearance of these lipid droplets.

Simms *et al.*(12) observed that fibroblasts cultured in medium containing fatty acid soaps tend to accumulate lipid droplets within their cytoplasm. This phenomenon was observed in our laboratory also but it was soon realized that the UFA droplets and S-serum droplets were not the same, neither morphologically nor in their physiological relation to the cell's metabolism, since the sterol tends to accumulate and is not utilized as a metabolic substrate by the cells. This is not true of droplets produced in presence of fatty acid as the following experiment demonstrates: Replicate cultures of mouse fibroblasts were grown on 10% S-serum and on a 10% NS-serum medium containing 25 mg % sodium oleate. After 48 hours exposure, at which time an abundance of lipid droplets were present in both cultures, the media were replaced by a 10% horse serum medium. Within 48 hours, the lipid droplets produced in the cultures containing UFA had disappeared. The droplets produced in the S-serum were still present, and remained even after 6 days.

In another experiment, insulin† (0.01 units per ml) and glucose (1 mg %) were added to oleate-containing and S-serum containing media before culture. Lipid droplet formation in the S-serum medium was unimpaired after 48 hours exposure while droplet formation in the oleate medium was prevented.

This latter was not unexpected, since Gordon and Cherkes(8) had previously found that fatty acid aggregation in rat adipose tissue *in vitro* did not occur in presence of insulin-glucose. It is significant, however, that this hormone-substrate combination did not affect lipid droplet formation in S-serum. Furthermore, heating the 2 media for 30 minutes at 89°C prior to inoculation prevents lipid droplet formation in S-serum medium but not in oleate-containing medium. These observations are listed in Table III and further support the belief that this phenomenon is not associated with UFA metabolism.

In a separate experiment, replicate cultures containing oleate and S-serum, respectively, were started. After 48 hours, the medium was removed from the cells and used for cultivating fresh cultures. Good growth was obtained in both groups of cultures, but the cells in the oleate medium now no longer produced lipid droplets, whereas lipid droplet formation in the medium containing S-serum was unimpaired. Then the now relatively depleted medium was again removed and used for cultivating additional fresh cells. Although growth was not exuberant, lipid droplets were still formed in the S-serum medium. From these results, it appears as though the active principle causing this phenomenon is not a metabolic substrate but is apparently acting in a catalytic manner.

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† Zinc free insulin furnished by Eli Lilly and Co.

Summary. Young chickens subjected to heat stress at 102°F for 24 hours elaborate a stress factor into their blood stream. When serum from these animals is used as an adjuvant in a serum-balanced salt solution medium, cells grown on this medium accumulate large numbers of small lipid droplets within their cytoplasm. The phenomenon is not related to substrate total fats, cholesterol or unesterified fatty acids. The droplet formation is associated with active cell growth and division and is not a function of degeneration. Evidence is presented to show that these droplets are, at least in part, steroid in nature and possibly cholesterol, and that they are produced intracellularly and escape by some means into the extra-cellular environment where they accumulate. They apparently are not used as metabolic substrate by the cells, but probably represent a by-product of the cell's altered lipid metabolism. It is possible that this type of phenomenon, functioning *in vivo* in animals under stress, may account in part for the hypercholesteremia of stress reported by Shafrir and Steinberg (4) and others. It also may furnish a clue to a source of extrahepatic endogenous cholesterol in the animal body. It is hoped that investigations of this type may renew interest in further use of cell culture techniques for detection of physiological alterations in the animal body.

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Conversion of Cholesterol to Bile Acids in Man.[**] (26916)

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Previous reports have shown that bile acids are final products of cholesterol degradation (for recent review see(1)), that rate of formation of bile acids is governed in part by reabsorption of bile acids from small intestine (2) and that half-lives of bile acids can be determined under physiological conditions in animals(3) as well as in man(4). Recently the rate of equilibration between cholesterol and bile acid pools has been studied(5,6). The present study was designed to determine rate of conversion of cholesterol to bile acids as well as sizes of cholesterol and bile acid pools in 2 hypercholesterolemic patients maintained solely on liquid formulas containing sterol-free triglycerides of known composition. By use of cholesterol-4-C^{14} and cholic acid-U-H^3 it was possible to determine rate of equilibrium between cholesterol and cholic acid and at the same time to measure cholic acid production.

Methods. 1. *Clinical.* Patient 1: 26 years, non-obese white male; 164 cm, 62 kg; hypercholesteremia; history of coronary insufficiency, but not of coronary occlusion or cardiac failure; arcus senilis, no xanthomatosis; no evidence of diabetes or other metabolic disorders; normal cholecystograms and EKG. Uneventful course on metabolic ward for 23 weeks.

Patient 2: 22 years, non-obese white female; 172 cm, 52 kg; hypercholesteremia and tendon xanthomatosis; no cardiovascular abnormalities, diabetes or other metabolic disorders; familial history of hypercholesteremia and early coronary disease. Uneventful course on metabolic ward for 26 weeks.

Both patients were fed solely on orally administered liquid formulas and maintained constant body weights (for complete regimen see ref. 7). Milk protein, fat and simple carbohydrates constituted 15, 40 and 45% of total calories. Entire dietary fat was synthetic triglycerides prepared from highly purified fatty acids, free of sterols (Table I). Daily intakes:
Patient 1–37.3 cal./kg, 103 g triglyceride A;
Patient 2–36.2 cal./kg, 87.6 g triglyceride B.

TABLE I. Composition of Synthetic Dietary Triglycerides.*

Fatty acids	Triglyceride A	Triglyceride B
Palmitic	57.4%	2.7%
Palmitoleic	.9	.3
Stearic	—	1.2
Oleic	3.6	60.3
Linoleic	35.5	35.5
Others	2.6	—
Iodine value	69	115
Free fatty acids	.2%	.3%
Hydroxyl value	1.6	0
Non-saponifiable	.4%	.4%
Trans acids	.7%	0
Conjugated dienes	.6%	.6%

* Prepared by Merck and Co., Rahway, N. J. (Dr. Erwin Schoenewalt). Fatty acid analyses by gas-liquid chromatography(8); other analyses performed by Procter and Gamble Co., Cincinnati, Ohio (Dr. Fred Mattson).

On day "zero" isotopic materials were administered by mouth: cholesterol-4-C^{14} (100 μC) and cholic acid-U-H^3 (5 μC) prepared by Wilzbach technic(9). Thereafter, bloods were drawn over a period of 3 weeks. Bile was aspirated through a duodenal tube after injection of 3 mg of cholecystokinin.[*] 2. *Chemical.* Serum lipids were separated and cholesterol isolated according to(10). Bile acids were isolated and separated(11,12,13) and analyzed by paper chromatography[†] (14). Isotope determinations were performed by gas-phase counting(15).

Results. The patients did well throughout the experimental period. Duodenal aspirations were most satisfactorily accomplished by introducing the tube the night before the aspiration. Total body weights remained unchanged. Values for serum lipid

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[*] We are indebted to Prof. E. Jorpes, Stockholm, for generous supply of cholecystokinin.

[†] We are indebted to Dr. J. Sjövall, Stockholm, for these analyses.

TABLE II. Serum Lipid Levels Before and During Isotope Study.*

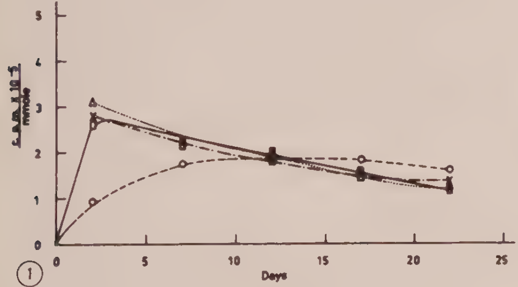
		Days before and after administration of labeled cholesterol and cholic acid										
		−27	−20	−13	−6	−3	+1	+8	+11	+15	+18	+22
Patient 1	Total cholesterol	350	341	346	348		350	305	312	295	287	280
"	Phospholipids	311	291	288	298		285	292	240	235	239	222
"	Triglycerides	236	311	345	344		334	146	255	165	261	236
Patient 2	Total cholesterol		270	285	255	238	267	279		271		273
"	Phospholipids		220	238	204	215	221	251		265		224
"	Triglycerides		163	195	138	151	196	179		159		162

* All values expressed as mg/100 ml serum.

levels(7) prior to and during the test period are given in Table II. In Patient 1 all levels decreased during the experimental period, while in Patient 2 there was no significant change.

Fig. 1 and 2 show the specific activities of serum cholesterol (free and esterified), bile cholesterol and cholic acid in the 2 patients. The specific activity curves for cholesterol fractions are not significantly different from those observed in normal cases(5,6). Cross-over of cholesterol and cholic acid specific activity curves occurred at 12 and 16 days, respectively, which is considerably later than the 4-5 days observed in healthy students of the same age(6). Fig. 3 and 4 are semi-logarithmic plots of specific activities of H^3-cholic acid from which pool size, half-lives and daily production have been calculated (Table III).

In 8 normal students previously studied the half-life of cholic acid was 2.8 days (1.2-4.2) and daily production of cholic acid 0.36 g (0.26-0.69 g). In the present cases the half-lives were 6.5 and 10.2 days, corresponding to daily productions of 0.087 g and 0.093 g, respectively. The total bile acid pool calculated from cholic acid pool and relative composition of bile was 1.70 and 2.30 g, essen-



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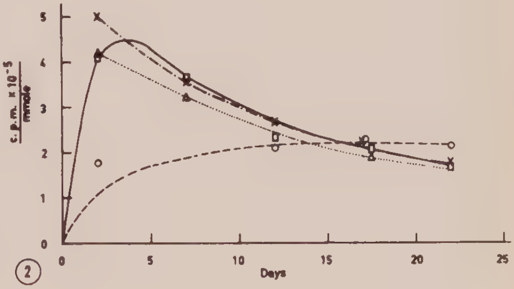

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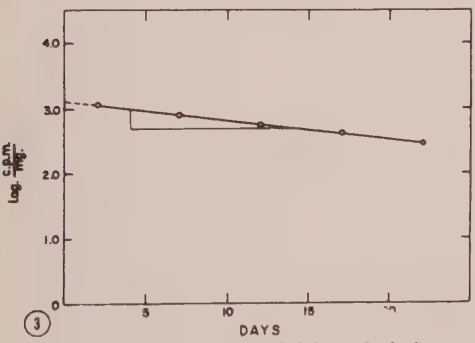

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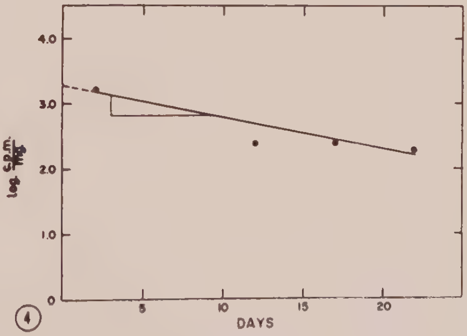

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FIG. 1. Specific activities of cholesterol and bile acids after administration of cholesterol-4-C^{14} (case 1). ×−•−•−×, serum free cholesterol; □——□, serum esterified cholesterol; △••••△, biliary cholesterol; ○−−−○, biliary cholic acid.

FIG. 2. Specific activities of cholesterol and bile acids after administration of cholesterol-4-C^{14} (case 2). For symbols, see Fig. 1.

FIG. 3. Semi-log plot of specific activities of biliary H^3-cholic acid (case 1).

FIG. 4. Semi-log plot of specific activities of biliary H^3-cholic acid (case 2).

TABLE III. Bile Acid Turnover Data(4).

	Half-life (days)	Pool size (g) Cholic acid	Pool size (g) Chenodeoxy-cholic acid	Pool size (g) Deoxycholic acid	Daily production of cholic acid (g)
Case 1	10.2	1.28	1.32	—	.087
" 2	6.5	.87	.50	.93	.093

tially within normal range (3.38 g, (1.88–4.97)(4)). Thus, decreased production of bile acids in these cases is caused mainly by prolongation of half-lives and not by decrease in pool size. It is interesting to observe that no detectable amount of deoxycholic acid was seen in Case 1, although this bile acid has been found in all other duodenal samples studied(16). Since this bile acid is formed from cholic acid by intestinal bacteria(17), it is possible that changes in intestinal flora caused by the diet are responsible for its absence in this case. However, Blomstrand (18) has noted the absence of deoxycholic acid in bile of a hypercholesteremic patient on *ad libitum* diet.

Previous experience has shown that complete analysis of fecal bile acids and sterols on regular diets presents great difficulties. The use of a synthetic sterol-free diet in these cases has been essential to analysis of fecal bile acids and sterols. These results will be published separately.

Discussion. Although it is interesting to speculate on the possible relation between decelerated cholesterol degradation and hypercholesterolemia in these patients, one should bear in mind that the formula diet *per se* may cause changes of bacterial flora that result in prolonged half-lives of bile acids. For example, it is known that germfree animals show changes similar to those observed here, *i.e.*, normal pool size and prolonged half-lives of bile acids(19). More definitive answers to the basic question (rates of production *vs.* degradation and excretion (20)) await extension of the present experimental approach in further patients fed diets causing alternately high and low serum cholesterol levels.

Summary. A technic is described for simultaneous determination in man of equilibration of cholesterol with bile acids and of bile acid turnover. Two hypercholesterolemic patients on formula diets containing synthetic fats showed prolonged bile acid half-lives with decelerated cholesterol degradation; cholic acid production was only 0.09 g per day (4-fold reduction from normal levels). Deoxycholic acid was totally absent from the bile in one case.

Technical assistance of Mr. Sven Jönsson is gratefully acknowledged.

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Mammary Tumor Development in Transplanted Hyperplastic Alveolar Nodules of the Mouse.* (26917)

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Hyperplastic alveolar nodules are commonly found in the mammary glands of old females from strains of mice, such as the mammary tumor virus (MTV)-infected C3H/Crgl, which have high incidences of mammary tumors. These nodules have been shown to be preneoplastic; when transplanted into the gland-free mammary fat pads of young virgin females, they develop mammary tumors more frequently and in less time than do samples of normal mammary tissue similarly transplanted(1,2).

Nodules are also found in large numbers in the mammary glands of old multiparous females from C3H sublines which do not possess demonstrable biologically active MTV (MTV-free). The nodules of the MTV-infected and the MTV-free sublines of C3H are similar in morphology, in ability to form hyperactive outgrowths following transplantation into gland-free mammary fat pads, and in the presence within their cells and lumina of virus-like particles as seen in the electron microscope(3). However, few females from the MTV-free C3H sublines develop mammary tumors.

Previous studies(4,5) have shown that cell populations derived from individual C3H/Crgl nodules have stable, inherent potentials of tumor development, and that these potentials may be either high or low. The occurrence in the C3H/Crgl females of some nodules with low potentials for mammary tumor development suggested that a higher proportion of such nodules in the MTV-free sublines could account for the observed low incidence of tumors. The present experiment was designed to test this possibility.

Materials and methods. Hyperplastic alveolar nodules from one strain (C3H/Crgl) and 2 sublines (C3Hf/Crgl and C3H/Crgl/2) were studied. The C3H/Crgl strain, which carries the MTV, is characterized by almost 100% tumor incidence in breeding females. In the C3Hf/Crgl subline, which was freed of biologically active MTV by foster-nursing on MTV-free females, approximately 20% of the breeding females develop mammary tumors at a much later average age. Another subline, C3H/Crgl/2, arose spontaneously in the C3H/Crgl strain, has no biologically active MTV, and is also characterized by a low incidence of mammary tumors at an advanced age.

Nodules from the mammary glands of non-pregnant, non-lactating, multiparous, tumor-bearing females of the C3H/Crgl strain were transplanted into the right and left mammary-gland-free inguinal mammary fat pads of 3-week-old C3H/Crgl females. Similarly, nodules from C3Hf/Crgl females were transplanted into C3Hf/Crgl hosts, and nodules from C3H/Crgl/2 females were transplanted into C3H/Crgl/2 hosts. Also, samples of adult normal C3H/Crgl mammary gland were transplanted into 3-week-old C3H/Crgl hosts. All hosts were maintained as virgins and were sacrificed 36-62 weeks after transplantation, or at prior tumor development. Some C3H/Crgl nodules were terminated without tumor development at ages earlier than 36 weeks when the nodule transplant in the host's other inguinal fat pad developed a tumor. At sacrifice, each inguinal fat pad containing a nodule transplant and any outgrowth was removed from the host, fixed in 10% formalin, stained with iron hematoxylin, and examined in methyl salicylate. Nodules from which no transplants were recovered were excluded from the data.

Results and discussion. Termination in weeks after transplantation for each nodule or sample of normal tissue is recorded in Table I. Twenty of 29 of the recovered C3H/Crgl nodules (69%) developed mammary tumors. However, only 2 of 15 C3Hf/

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TABLE I. Distribution in Time of the Appearance of Tumors in C3H/Crgl Normal Adult Mammary Gland and in C3H/Crgl, C3Hf/Crgl, and C3H/Crgl/2 Nodules Transplanted Respectively into C3H/Crgl, C3Hf/Crgl, and C3H/Crgl/2 3-Week-Old Females. Tumor development, or termination without tumor development, for each nodule or sample of normal tissue is recorded in weeks after transplantation.

Tissue and strain of origin		Weeks after transplantation					
		9–15	16–22	23–29	30–36	37–43	44 & over
C3H/Crgl nodules	No. with tumor	6	4	10	—	—	—
	No. without tumor	2	1	3	1	—	2
C3Hf/Crgl nodules	No. with tumor	1	—	—	—	—	1
	No. without tumor	—	—	4	1	—	8
C3H/Crgl/2 nodules	No. with tumor	—	—	1	—	—	—
	No. without tumor	—	—	2	4	4	6
C3H/Crgl normal mammary gland	No. with tumor	1	—	—	2		
	No. without tumor	—	3	9	20		

Crgl nodules (13%) and only one of 17 C3H/Crgl/2 nodules (6%) developed mammary tumors. Three of 32 transplants of normal C3H/Crgl mammary tissue (9%) developed mammary tumors within 36 weeks. Thus, more of the C3H/Crgl nodules developed mammary tumors in less time than either the C3Hf/Crgl or the C3H/Crgl/2 nodules. In fact, the nodules from the 2 MTV-free sublines developed no more tumors than did samples of normal C3H/Crgl mammary tissue.

Outgrowths from several of the C3Hf/Crgl and C3H/Crgl/2 nodules have been retransplanted, and are being maintained serially. For 3 generations the C3Hf/Crgl outgrowth which developed a mammary tumor within 15 weeks (Table I) has continued to show a high tumor potential. In addition, 5 C3Hf/Crgl and 6 C3H/Crgl/2 nodules which did not produce tumors in the original experiment have retained that characteristic following retransplantation of the outgrowths. Thus, in the C3Hf/Crgl and C3H/Crgl/2 sublines, as in the C3H/Crgl strain(4,5), the potential for tumor development is an inherent character of the cell populations comprising nodules.

Preliminary data from an experiment in progress indicate that simple exposure to the MTV (achieved by transplanting outgrowths from C3Hf/Crgl and C3H/Crgl/2 nodules into MTV-infected hosts) is not sufficient to create a tumor potential in these nodule outgrowths similar to that seen in most C3H/Crgl nodules. The 13 non-tumor-producing C3Hf/Crgl nodules (Table I) were retransplanted into 3-week-old C3H/Crgl females. The nodule outgrowths have been maintained in these MTV-infected hosts for at least 31 weeks, and 11 of them have developed no mammary tumors. None of the outgrowths from 11 C3H/Crgl/2 nodules similarly retransplanted into C3H/Crgl females have developed mammary tumors after at least 25 weeks. This is in contrast with the development of mammary tumors in 69% of the C3H/Crgl nodules within 29 weeks in the original experiment.

Although the hyperplastic alveolar nodules from the MTV-infected C3H/Crgl strain and the MTV-free C3H sublines are similar in the several characteristics mentioned above, the population of nodules created in the presence of the MTV contains more nodules with a high capacity for tumor development than do the nodule populations created in the absence of the MTV.

Summary. Nodules from adult C3H/Crgl females produce a high incidence of mammary tumors following transplantation into young females. On the other hand, nodules from two C3H sublines which lack demonstrable biologically active mammary tumor virus (MTV) rarely develop mammary tumors when similarly transplanted. Simple exposure to MTV (achieved by transplanting nodular outgrowth from the MTV-free sublines into MTV-infected C3H/Crgl hosts) does not create tumor potentials in these outgrowths equivalent to those seen in the C3H/Crgl population of nodules. It is concluded that a different population of nodules (with

regard to tumor potential) is formed in the absence of the MTV than in its presence.

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Blockade of Gonadotrophin Release for Ovulation in the Hen following Stimulation with Stainless Steel Electrodes. (26918)

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The release of pituitary gonadotrophin for ovulation following electrical stimulation of certain regions of the brain has been demonstrated in several mammalian species(1). Similar responses appear not to have been described in birds. The experiments reported here were undertaken to ascertain the possible effectiveness of stimulation in the induction of ovulation prematurely in the hen. Our procedures proved of no value in this respect, but unexpectedly resulted in apparent postponement of ovulation for some hours beyond the time of its normal occurrence in a substantial proportion of treated birds. These experiments, some results of which have been reported in abstract(2) are described here. Evidence is presented also for the conclusion that the observed delays in ovulation are dependent upon delay in, or blockade of, release of the ovulation-inducing hormone (OIH) in quantities adequate to assure ovulation.

Procedures. All experiments were carried out with White Leghorn hens in first or second years of production, individually caged in batteries under electric lights from 6:00 a.m. to 8:00 p.m., and with free access to feed and water. Hourly records of lay, routinely maintained from 8:00 a. m. through 4:00 p. m., served to identify hens regularly ovulating sequences of two or three members and failing to ovulate on but a single day between repeated sequences(3). Under conditions prevailing in this Laboratory, ovulation of the first or C_1 follicle in such sequences takes place at about 6:00 a.m. The administration of exogenous luteinizing preparations or of progesterone at around 4:00 p. m. of the day preceding ovulation of the C_1 follicle (the day of "missed" ovulation between sequences) forces its ovulation some 7 to 8 hours thereafter, or 6 to 7 hours prematurely. On the basis of these well-documented normal and experimental relationships(3), electrical stimulation was applied between 3:00 and 5:00 p. m. for effect on C_1 follicles normally destined to ovulate at about 6:00 a.m. of the following day.

The hour of ovulation was estimated by routine digital palpation of the oviducal egg (4), supplemented by observations at autopsy. Two orders of delayed ovulations were recognized; (i) presumptively delayed ovulations, completed before autopsy; and (ii) definitively delayed ovulations, based on presence of the intact C_1 follicle and complete absence of follicular atresia at time of autopsy.

Using the stereotactic procedures described by Ralph(5), bipolar stainless steel electrodes were placed bilaterally in the diencephalon, 1.0 mm on either side of the midline. The electrodes consisted of 2 parallel lengths of 29 gauge wire, each completely insulated except for a 0.5 mm ring at the tip, with the exposed ends separated by 0.5 mm.

The standard stimulus consisted of biphasic 1 msc pulse pairs, 100 cps, applied during

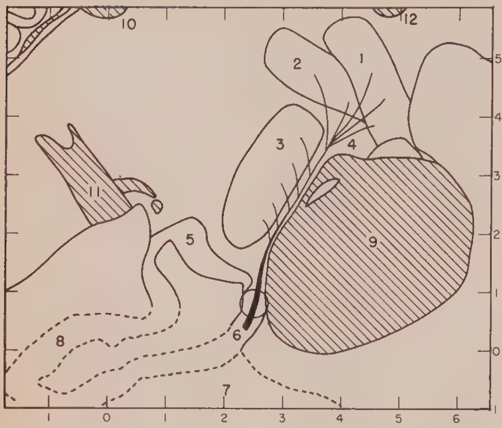

FIG. 1. Diagram of a parasagittal section of chicken diencephalon showing 1-mm stereotaxic coordinates. Dotted lines indicate approximate position of the hypophysis. 1, preoptic paraventricular nucleus; 2, magnocellular paraventricular nucleus; 3, lateral hypothalamic nucleus; 4, anterior hypophyseal tract; 5, tubero-mammillary nucleus; 6, median eminence; 7, anterior pituitary; 8, posterior pituitary; 9, optic chiasma; 10, posterior commissure; 11, oculomotor nerve; 12, tractus septomesencephalicus. (Based on C. L. Ralph, *Am. J. Physiol.*, 1959, v197, 1279.)

alternate 15 second intervals for a period of 10 minutes. The current was delivered from a Grass model S-4 stimulator and was passed through a model UTC - M 1019 pulse transformer. Current was continuously monitored oscilloscopically and was maintained at approximately 200 μA.

In an exploratory study including 67 birds, stimulation was applied routinely for 10 minutes with stainless steel electrodes in the preoptic hypothalamus, along the dorso-caudal surface of the optic chiasma in the anterior hypophyseal tract, or in the region of the median eminence. Stimulation in regions rostral to the median eminence had no effect on subsequent ovulation. Results of stimulation in a region of the median eminence at the level of the anterior hypophyseal tract (Fig. 1) were unexpected and of particular interest.

Control experiments included stimulation with platinum electrodes and placement of stainless steel and platinum electrodes for 10 minutes without passage of current. The platinum electrodes were of the same construction as the stainless steel electrodes, and were used under identical conditions in the stimulation experiments.

To verify the placement of electrodes at or close to the selected coordinate intercepts (Fig. 1), thionin stained frozen sections were made of the brains, selected at random, of approximately half the treated birds. Sagittal reconstructions were made for purposes of localization.

The luteinizing hormone (LH) preparation* used to test ovulability of the C_1 follicle following stimulation was dissolved in distilled water, 5 mg/ml and injected intravenously. In similar tests, progesterone was administered intravenously or intramuscularly. The steroid was dissolved in propylene glycol, 5 mg/ml, for intravenous use. Progesterone in corn oil, 5 mg/ml, served for intramuscular injections.

Results. The 18 hens of Group A of Table I were sacrificed 24 hours following stimulation, or 10 hours later than the hour of normally expected ovulation. Ovulation occurred at the normal hour in 9 birds. In 6, ovulation was delayed, but had taken place before the hour of autopsy. As judged by palpation records and stage of egg formation at autopsy, ovulation was delayed by some 3 to 6 or 7 hours in 5 of these hens. The ovulated yolk was found in the body cavity of the sixth hen, so located as to indicate its ovulation some hours before autopsy. The C_1 follicles of the remaining 3 hens were intact.

The finding of a yolk free in the body cavity of one hen of Group A led to the suspicion that ovulation might have occurred at the normal hour in some of the 6 hens ovulating before autopsy, the apparent delays in ovulation signifying impaired oviducal function, *e.g.*, failure to engulf the yolks within the usual brief intervals following ovulation. If this were so, an increasing proportion of yolks should be expected in body cavities as the interval from the hour of normal ovulation to autopsy was decreased. Hens were accordingly sacrificed 7 and 4 hours following the hour of normally expected ovulation (Groups B and C respectively, Table I). While 2 hens of Group C did carry yolks in body cavities, none were found in the hens of Group B.

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* "PLH" of Armour Pharmaceutical Co., Kankakee, Ill.

TABLE I. Follicular Responses Following Stimulation 14 Hours before Expected Normal Ovulation.

Group	Stim'l'n to autopsy, hr	Normal ovulation time to autopsy, hr	No. hens	Ovulations*				Yolk in body cavity
				No. normal	No. delayed			
					P†	D†	Total	No. hens
A	24	10	18	9	6	3	9	1
B	21	7	16	7	3	6	9	0
C	18	4	18	7	3	7	10	2
		Totals	52	23	12	16	28	3

Follicular atresia was not observed in any hen.

* Ovulation was induced prematurely in one of the 18 hens of group C.

† P, presumptively delayed ovulations; D, definitively delayed ovulations.

The "average" incidence of yolks in body cavities—one per group—is clearly of little significance beyond suggesting that the oviduct of an occasional hen may fail to engulf the ovulated yolk.

Other results recorded in Table I bear more directly on the fact of actual delay in presumptively delayed ovulations. The incidence of normal ovulations is about the same in all 3 groups, as is the incidence (total) of delayed ovulations. In Groups B and C, however, the proportion of presumptively to definitively delayed ovulations is nearly the reverse of that seen in Group A. The inference to be drawn from these relationships is that some follicles not ovulated at 4 hours (Group C) or even at 7 hours (Group B) later than the hour of normally expected ovulation would have ovulated between 4 and 10 or 7 and 10 hours thereafter (cf. Group A). There could be no question concerning actual delay in the hour of ovulation of these follicles. If this be so, it seems probable that all ovulations of the "presumptively delayed" category, except the 3 resulting in yolks in body cavities, were in fact delayed to the extent indicated by palpation records and autopsy findings.

If any doubt remains as to delay of ovulation in hens ovulating before autopsy, there need be none concerning those hens in which ovulation had not occurred at autopsy, the definitively delayed ovulations of Table I. The absence of follicular atresia, even at 24 hours after stimulation, is especially worthy of note in this connection.

It was recognized that some proportion of delayed ovulations of Table I might result from palpation itself, or from an uncommon spread in the hour of normal ovulation. Fifty-six control hens ovulating in cycles comparable with those of the stimulated birds accordingly were palpated in the same routine. Ovulation occurred within normally expected limits in all hens.

Stimulation with platinum electrodes, or placement of either stainless steel or platinum electrodes without passage of current, all failed to delay ovulation (Table II). Placement of stainless steel electrodes without passage of current resulted, however, in the appearance of follicular atresia in those hens failing to ovulate at the normally expected hour, a condition not encountered in hens of the other groups nor in any hen subjected to stimulation with steel electrodes (Table I).

Follicular responses to LH and to progesterone administered at differing intervals following stimulation are recorded in Table III. Ovulation occurred in all of 17 hens injected with LH 6 hours following stimulation, *i.e.*, at about the hour of normal OIH release. Ovulation would have occurred in about half of these hens in response to normal release of OIH (Table I); the injected LH thus induced ovulation in the remaining half or

TABLE II. Effects of Stimulation with Platinum Electrodes and of Electrode Placement on Hour of Ovulation of C_1 Follicle.

Electrodes	Current passed	No. hens	No. ovulations		Atresia, No. hens
			Normal	Delayed	
Platinum	Yes	15	15	0	0
Steel	No	27	21	0	6
Platinum	"	13	13	0	0

TABLE III. Follicular Responses to LH and to Progesterone (Pgst) Administered Systemically at Stated Intervals Following Stimulation with Stainless Steel Electrodes.

Substance	Route*	Quantity, mg	Stim'l'n to inj., hr	No. hens	No. ovulations		Atresia, No. hens
					Total	Forced	
LH	Intrav.	2.5	6	17	17	†	0
”	”	2.5	18	7	5	2/4	5
Pgst	Intrav.	10	6	10	5	None	5
”	Intramusc.	1	6	11	11	†	0
”	”	1	0	14	14	12	2

* Intrav., intravenous; Intramusc., intramuscular. † About half of total; see text.

thereabouts in which delay was expected. No follicular atresia was seen in the ovaries of these birds at autopsy, 24 hours following stimulation.

The 7 hens of a second group were injected with LH 18 hours following stimulation. Ovulation was induced in only 2 of 4 hens in which it had not occurred before injection. Curiously, the C_1 follicles of the 2 hens failing to respond to LH showed no signs of atresia at autopsy 42 hours following stimulation, although most follicles of intermediate size were atretic.

Progesterone, administered intravenously 6 hours after stimulation, apparently failed to induce ovulation in any of the hens in which delay might have been expected (Table III). In contrast, ovulation occurred in all hens injected intramuscularly 6 hours after stimulation, and in about half of these progesterone must have induced the ovulation. Progesterone was similarly administered to hens of the last group of Table III immediately following stimulation, thus about 6 hours before normal release of OIH. Ovulation was induced in 12 of the 14 hens. Yolks were found in the body cavities of the 2 remaining hens upon autopsy 24 hours after stimulation, but times of ovulation could not be known. Atresia was seen in the follicles of only 2 hens; ovulation had been forced in both birds.

A final experiment was carried out to ascertain the fate of C_1 follicles which appeared ovulable 18 to 24 hours following stimulation, as were those of the 16 hens of Table I in which ovulation had not occurred at the hour of autopsy. Twenty-one hens were subjected to stimulation in the usual manner. Nine of the 21, a rather high proportion, were found not to carry a palpable egg 24 hours later. These 9 birds were palpated routinely the next day, and were then sacrificed at 4:00 p.m., 48 hours following stimulation. In 4 hens, palpation records and post-mortem findings established the occurrence of ovulation, at about 6:00 a.m., of the C_1 follicles whose ovulation had been delayed by stimulation. Follicular atresia had supervened in the remaining 5 hens.

Discussion. The observation that delays in ovulation in hens of the first group of Table I were of the order of 3 to 6 hours, or greater than even 10 hours, but not between some 6 and 10 or more hours, was indicative clearly of delay in or blockade of OIH release. The results recorded in Table III confirm this. Of particular interest is the response to progesterone, for this steroid acts through central nervous channels rather than on either the anterior pituitary gland or the follicle(3,6). Perhaps the most convincing evidence of blockade of OIH release by stimulation is afforded, however, by those 4 hens in which ovulation occurred without further intervention 24 hours later than would have been expected in absence of stimulation.

The timing relationships between the hour of stimulation and normal or delayed OIH release are represented in Fig. 2, which includes the final hours of the terminal day of one cycle and day 1 of a succeeding cycle. The open period embraces those 6 to 8 hours of the 24 within which OIH release may occur; the closed period includes the 16 to 18 hours of the 24 within which OIH release does not normally occur. The onset of OIH release, R_1, for ovulation of the C_1 follicle takes place about 10:00 p. m., some 2 hours following the onset of darkness. If an ap-

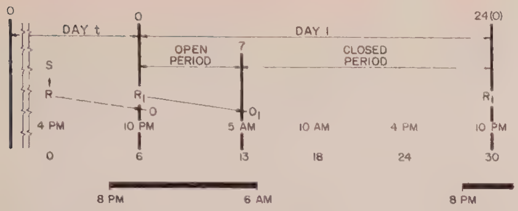

FIG. 2. Schematic representation of timing relationships involved in normal, blocked or advanced release of OIH for ovulation of the C_1 follicle. Open period: hours of the 24 within which OIH may be released. Closed period: hours of the 24 during which OIH is not normally released. R_1 at hour 0 of cycle day 1, normal OIH release for ovulation, O_1, of the C_1 follicle; R_1 at hour 24 of day 1, OIH release for ovulation of the C_1 follicle following delay of 24 hr (blockade through one open period). R, induced release of OIH in response to stimulus, S, at 4:00 P.M., or 6 hr before onset of cycle day 1. The horizontal bars at bottom of the figure represent the hours of darkness.

propriate stimulus, such as progesterone, evokes OIH release at 4:00 p. m., ovulation is correspondingly advanced.

It is clear that if electrical stimulation at 4:00 p. m. is to delay OIH release at all, it must act, directly or indirectly, at least some 6 hours later (Fig. 2). OIH releases delayed by 3 to 6 hours thus call for effective action 9 to 12 hours following stimulation. But it is clear also that duration of effect of stimulation need not exceed some 12 or 14 hours to account for the continuing delay in OIH release, since by these hours suppressive or inhibitory factors associated with the closed period come into play. With the termination of this period and onset of the succeeding open period, the blockade of OIH release is or may be ended, as was attested by OIH release in the 4 hens spoken of earlier.

Everett and Radford(7,8) have demonstrated very recently that the release of gonadotrophin for ovulation induced in the rat by brief stimulation with stainless steel electrodes is in fact mediated by electrolytically deposited iron. The delay in or blockade of OIH release in the hen following stimulation with stainless steel electrodes appears to be mediated similarly, for the effect cannot be produced by comparable stimulation with platinum electrodes nor by simple placement of electrodes. The physical basis for prolonged effect following stimulation for a brief interval thus seems to be established.

The physiological processes through which "irritative deposits" of iron may delay or block OIH release remain a matter of conjecture. A simple mechanical obstruction to transmission of nervous impulses or to passage of neurohumoral substances over fibers of the anterior hypophyseal tract seems improbable in view of the absence of atresia in the follicles of any of the 52 hens subjected only to stimulation (Table I). The fact seems the more notable in view of the advanced follicular atresia seen in some hens following the secondary treatments recorded in Table III, or placement of electrodes without passage of current (Table II). It is true that atresia was observed in 5 of the 9 hens autopsied 48 hours following stimulation alone, but this might well have been the result of OIH release at subovulatory levels, some 30 hours following stimulation (Fig. 2). In any event, follicular integrity was maintained in the remaining 4 hens in which OIH release was blocked over the initial open period and through the succeeding closed period of the cycle.

Stimulation in the tuber cinereum, bilaterally just above the median eminence, induces release of gonadotrophin for ovulation in the rat(9). Under the conditions of our experiments, stimulation in a comparable region of the hen's brain evokes the contrary response. But as in the rat, we might reasonably expect the differing response in the hen to be imposed by some positive (rather than impaired) function of the anterior pituitary. One possibility is that stimulation causes the secretion of gonadotrophin(s) at levels conducive to maintenance of estrogen concentrations capable of suppressing OIH release. Estradiol benzoate may delay ovulation of the second follicle of the ring dove's sequence by about 24 hours(10), and delays ovulation of the second follicle of the hen's 2 or 3 member sequence(11). It must be noted, however, that delay in the hour of ovulation of C_1 follicles was seldom achieved. Intramuscular injection at appropriate times of anterior pituitary preparations, or of LH or of LH + FSH preparations of chicken origin, effectively blocks the release of OIH for effect on the second follicle of the hen's 2 member sequence(12). It was suggested that the blockade of OIH release by the same gonadotrophic preparation (LH) so effective in forcing ovulation under differing conditions might be the outcome of increased blood estrogen levels, but we have no direct evidence that this is so. It would be of much interest to know estrogen levels not only before and after gonadotrophin administration, but also during the course of the specific activity consequent upon electrical stimulation in the hen.

Our results do not exclude the possibility that stimulation at other regions of the hen's brain, or with current of differing parameters, may induce OIH release in the hen as it does in some mammalian species. The single forced ovulation recorded in Table I may be of some import in this connection, since LH release at 4:00 p. m. of the terminal day of the cycle is not encountered normally. The differing response of the hen to stimulation may nevertheless betoken differences in organization and function of nervous or endocrine factors in birds and mammals. It is of interest in this connection that some barbiturates regularly blocking OIH release in the rat may induce OIH release prematurely in the hen(13).

Summary. Bilateral stimulation with bipolar stainless steel electrodes was applied for 10 minutes in a selected region of the median eminence of the hen, 14 hours before expected normal ovulation of the C_1 follicle. In 28 of 52 hens, ovulation was delayed by 3 to 10 hours or more. The delays in ovulation were shown to arise out of delays in or blockade of the release of ovulation-inducing hormone (OIH). The effects of stimulation on delay of OIH release may extend over a period of 12 to 14 hours. Stimulation with platinum electrodes or placement of steel or platinum electrodes without passage of current failed to delay OIH release. The prolonged effects of stimulation with stainless steel electrodes are consistent with the conclusion of Everett and Radford that electrolytically deposited iron may act irritatively long after application of the stimulatory current.

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Discrimination in Intestinal Absorption of Strontium and Calcium.* (26919)

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The preliminary results here reported represent an effort to study the effect of calcium on absorption of trace levels of radiostrontium from the intestinal tract of rats using a method which focuses attention on the absorption process. To eliminate the influence of excretory and bone deposition processes, many recent studies of intestinal absorption have been conducted *in vitro,* using the everted intestinal-sac technic described by

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Wilson and Wiseman(1). Schachter and coworkers, in a series of papers, have used this technic to study factors which affect the transport of calcium from the small intestine of the rat(2,3,4). An active transport system seems to be involved in the absorption of a part of the calcium, and this system appears not to be involved in strontium absorption. Wasserman has more specifically compared the absorption of strontium and calcium, using the intestinal-sac technic, and has shown that strontium absorption, unlike calcium absorption, is insensitive to prior history of dietary calcium levels(5).

Curran and Solomon have devised a technic of perfusing a segment of the intestine, *in vivo*,(6) which they have used in studying absorption of calcium and strontium in rats. This technic is a more physiological approach to the problem than the intestinal-sac technic and provides a measure of intestinal absorption which is for most purposes not significantly affected by the subsequent fate of the absorbed material in the animal. Using this perfusion technic, Dumont, Curran and Solomon studied the absorption of both calcium and strontium as a function of their concentration in the lumen of the intestine(7). Their observations on the absorption of strontium were compatible with a passive diffusion process. The absorption of calcium showed evidence, at higher intraluminal calcium levels, of an active transport process. These studies involved perfusion of the entire ileum plus a portion of the jejunum. Wasserman *et al.* have recently reported the results of a similar study, with calcium only which, however, involved perfusion of a segment of the duodenum(8). The active transport of calcium was much more evident in these duodenal preparations, as would have been predicted from intestinal-sac results(2,9).

The present studies were not designed to elucidate absorption mechanisms but to measure the discrimination between strontium and calcium in the process of absorption from the intestine, and to determine the effect on this discrimination of varying the level of calcium intake. Data were also obtained on the movement of intravenously injected strontium and calcium into the intestine.

Methods. Female albino rats of the Sprague-Dawley strain were fasted for 24 hours in preparation for the perfusion studies. They had previously been maintained on Purina Lab Chow (calcium content, 1.3%). The entire small intestine was perfused under pentobarbital anesthesia. The inflow cannula, inserted through the mouth, was tied in place in the upper duodenum, through an abdominal incision. The outflow cannula was inserted into the small intestine and tied in place at the ileo-cecal junction. The intestine was disturbed as little as possible and left in the animal during perfusion. The incision was covered with saline-moistened gauze and temperature of the animal maintained with heat lamps. In one series of experiments the large intestine was cannulated in a similar manner and perfused from cecum to anus.

The perfusion solution was maintained at body temperature, and circulated by means of a peristaltic action pump. The perfusion rate employed was approximately 1.0 ml/min and the perfusion pressure did not exceed about 7 cm of water. The total perfusion period in all experiments was 100 minutes. Preliminary experiments had shown that rate of absorption of Sr^{85} or Ca^{45} was independent of rate of perfusion in the range from 0.5 to 1.5 ml/min, and remained reasonably constant for perfusion periods of up to 100 minutes. The perfusion solutions were 5 mM in KCl, 20 mM in glucose, and 0, 1, 5 or 25 mM in $CaCl_2$. Strontium-85 was added as a solution of the nitrate; Ca^{45}, as a solution of the chloride. The quantity of non-radioactive strontium and calcium associated with the radioisotopes in no case exceeded a final concentration of 1 μg/ml in the perfusion solution. Total osmolarity of all solutions was adjusted to 300 osmM with NaCl. pH was adjusted to approximately 7 with NaOH.

In the first series of experiments, Sr^{85} and Ca^{45} tracers were added to the perfusion solutions at levels of approximately 0.1 μc/ml. The animals were first perfused for 25 minutes with a solution containing no radioisotopes; the same solution, but containing Sr^{85} and Ca^{45}, was continued for the next 50 minutes, followed for a final 25-minute flush with the radioisotope-free solution. Animals were sacrificed at conclusion of the perfusion and radioisotope content determined in the total animal (exclusive of small intestine) and in the effluent perfusion solution. In the second series of experiments, Sr^{85} and Ca^{45} in amounts of approximately 2 μc, (approximately 7 μg non-radioactive calcium, less than 1 μg non-radioactive strontium) were injected into the tail vein 50 minutes after the start of perfusion. The perfusion was continued for an additional 50 minutes following which the animals were sacrificed and radioisotope content determined in the total animal (exclusive of tail), in the segment of tail adjacent to the injection site, and in the effluent perfusion solution. If more than 2% of the injected radioisotope was found at injection site at the end of experiment, the data were discarded.

Sr^{85} was determined by gamma-scintillation counting in a sodium-iodide well-type counter. Following determination of Sr^{85}, the samples were ashed and aliquots counted for the Ca^{45} betas in a thin-window proportional counter. The Sr^{89} impurity in the Sr^{85} was shown to contribute insignificantly to the Ca^{45} count.

Results and discussion. Data on absorption of Sr^{85} and Ca^{45} from the perfused small intestine as determined from analyses on the total animal are shown in Table I. When expressed as per cent of total radioisotope administered, absorption of both Sr^{85} and Ca^{45} is inhibited by addition of calcium to the perfusion solution. In terms of total calcium absorbed, there is, of course, an increase as the calcium level in the perfusate is increased. The absolute rate of calcium absorption (Table I), is calculated from the radioisotope data on the assumption that the Ca^{45}/total calcium ratio in the solution perfusing the intestine remained constant during transit. This is not strictly true, since unlabeled endogenous calcium is excreted into the intestine throughout the perfusion. Rates of total calcium absorption are in reasonable agreement with those obtained in earlier studies, being lower by a factor of 2 at the 1 mM calcium level than the duodenal rate measured by Wasserman *et al.*(8); and somewhat higher at the 1 and 5 mM calcium level than the rates measured by Dumont *et al.* in the ileum and jejunum(7). The rate at 25 mM calcium is considerably lower than the average value of 0.9 μM/hr cm measured by Dumont *et al.* At this high calcium level the movement of calcium into the intestine has been shown to be appreciable(7), and the assumption of constant specific activity used in estimation of our total calcium absorption values is apt to introduce the greatest error.

TABLE I. Absorption of Sr^{85} and Ca^{45} from the Perfused Small Intestine of the Rat (Average Values from 6 Rats ± Stand. Dev.).

Conc. of Ca in perfusate (mM/l)	% of perfused radioisotope absorbed			Total Ca absorbed* (μM/hr cm)
	Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}	
0	4.3 ±.6	14.9 ± 2.3	.29 ±.04	—
1	3.2 ±.6	7.3 ± 1.6	.45 ±.04	.04
5	2.6 ±.3	4.8 ± .4	.54 ±.06	.14
25	1.4 ±.1	2.2 ± .2	.63 ±.04	.33

* Estimated on the assumption of constant Ca^{45}/Ca^{40} ratio in solution passing through intestine.

Of more significance to the present investigation are the ratios of Sr^{85}/Ca^{45} absorbed. These numbers accurately reflect the relative behavior of exogenous calcium and strontium and indicate not only a discrimination against strontium in the absorption process, but a discrimination which changes as a function of calcium intake. These results are in accord with observations on continuous feeding of radiostrontium to rats, where the ultimate deposition of strontium and calcium in bone showed a similar pattern of variable discrimination as a function of dietary calcium level (10,11). The inference may be drawn that the pattern of strontium and calcium deposition is determined largely by the discrimination which occurs in the absorption process. Discrimination may occur in other physiological processes. It would appear, in fact, that greater discrimination occurs in the renal excretory processes(11). However, the discrimination which occurs in absorption from the intestine is totally effective, in that all ingested strontium is subjected to this process; whereas, that which occurs in the kidney affects only that fraction of the ingested strontium which ultimately passes through this organ.

The results listed in Table I might be in-

TABLE II. Excretion of Intravenously Injected Sr^{85} and Ca^{45} into the Perfused Intestine of the Rat (Average Values from 3 to 5 Rats ± Stand. Dev.).

Segment perfused	Perfusate [Ca] (mM)	% of inj. radioisotope excreted		
		Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}
Small intestine	0	3.1 ± .1	2.6 ± .3	1.2 ±.2
	1	4.2 ±1.2	4.1 ±1.1	1.0 ±.1
	5	2.6 ± .3	2.5 ± .3	1.0 ±.2
	25	1.9 ± .2	1.9 ± .2	1.0 ±.2
Large intestine	1	.5 ± .3	.4 ± .3	1.3 ±.4

terpreted in a very different manner if a substantial portion of the absorbed radioisotopes were re-excreted into the intestine. Thus, one might assume that the intestine was completely permeable to both strontium and calcium, and that the observed discrimination was due to selective binding of calcium on bone. That such is not the case was shown in a second experiment in which Sr^{85} and Ca^{45} were introduced into the blood and their appearance measured in the perfusate. The results (Table II) indicate that no very substantial fraction of Sr^{85} or Ca^{45} reaching the bloodstream is excreted into the intestine. The apparent decrease in movement of Sr^{85} and Ca^{45} into the intestine with increasing levels of calcium in the perfusion solution was unexpected, in view of the reported increase in movement of calcium into the intestine under these conditions(7). The explanation would seem to lie in the fact that the Ca^{45} in this experiment cannot be considered as a tracer for blood calcium, and while the ultimate effect of an increased calcium load in the blood may be to return a larger portion of calcium to the intestine, the immediate effect would be to accelerate the deposition of this calcium on bone, where the Ca^{45} label would be diluted with the freely exchangeable calcium of bone.

Of particular interest in Table II are the Sr^{85}/Ca^{45} ratios in the perfusate, which are in no case significantly different from unity. There would appear to be no discrimination between strontium and calcium in this process of excretion into the intestine. To establish this point with certainty it would have been necessary to measure blood levels of Sr^{85} and Ca^{45} during the course of perfusion. This was not done. Some further evidence for a lack of discrimination in this process was provided, however, by the fact that the perfusate when collected and analyzed in successive 10 ml portions, showed no variation in the ratio of Sr^{85}/Ca^{45}, with time of collection, although the total quantities of radioisotopes fell sharply after collection of the first 10 to 30 ml.

The very small amount of Sr^{85} and Ca^{45} excreted into the large intestine (Table II) indicates that no appreciable error was introduced by excluding this section of the intestine from the remainder of the study.

A word of caution is in order concerning the interpretation of these experiments. Although the technic of *in situ* perfusion of the intestine is clearly more physiological than the study *in vitro* of an intestinal preparation, there remain important non-physiological features. The composition of the perfusing solution is rather markedly different from the normal content of the intestine, and the uniform rate of passage through all segments of the tract is unlike the situation which normally pertains. A more detailed study of the problem would involve perfusion of separate segments of the intestine, weighting the results from each segment in the light of normal rates of passage through that segment (12). Despite these limitations the data indicate, at least semi-quantitatively, the importance of the absorption process in the discrimination between strontium and calcium and the variability in this discrimination which occurs as a function of calcium intake level.

Summary. Absorption of Sr^{85} and Ca^{45} was studied in the rat by the technic of *in vivo* intestinal perfusion. With no calcium added to the perfusion solution the percentage of Sr^{85} absorbed was about 0.3 that of Ca^{45}. With increasing levels of calcium in the perfusion solution, the percentage of Sr^{85} and Ca^{45} absorbed decreased; however, the ratio of percentage absorption of Sr^{85} to Ca^{45} increased, reaching a value of about 0.6 in 25 mM calcium solution. There was no evidence of discrimination in the reverse movement of Sr^{85} and Ca^{45} from blood to intestine. The discrimination which occurs in the absorption

process would appear to be a dominant factor in determining over-all strontium calcium relationships.

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Coenzyme Q Concentration in Proliferative Lesions of Liver.* (26920)

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Histochemical studies of the succinic dehydrogenase system using tetrazolium salt reduction have shown a decrease of succinate-tetrazolium reductase activity in a number of benign and malignant proliferative lesions (1,2,3). Recent work employing the tetrazolium salt, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), has indicated that a member of the coenzyme Q group of quinones serves as an intermediate electron transport agent in the succinate-INT reductase reaction, and that in many tissues this system is not saturated with respect to quinone(4,5,6). Accordingly, a decrease in succinate-INT reductase activity might be due to either a decrease in activity of the primary dehydrogenase, a lack of availability of quinone, or some combination of the two effects. An effort was made to ascertain which of these alternatives exists in regenerating liver and the Novikoff hepatoma by addition of coenzyme Q_{10} to the reaction mixture. It was found that with frozen sections of the Novikoff hepatoma addition of coenzyme Q_{10} to the succinate-INT system resulted in a considerably greater enhancement of reductase activity than occurs in normal liver(5). This suggested that the coenzyme Q content of this neoplasm might be relatively low. In contrast, in regenerating liver addition of coenzyme Q_{10} to the reaction mixture caused an enhancement of activity of approximately the same order of magnitude as that of normal liver. These findings suggested that the coenzyme Q content of regenerating liver was not significantly reduced, but that some other factor was causing the decrease in activity observed. Since the enhancement of activity of the succinate-INT reductase activity by addition of coenzyme Q_{10} to the reaction mixture can be considered at best as supplying only indirect evidence of the relative amount of coenzyme Q available for the reductase reaction, direct quantitative studies of the coenzyme Q content of these tissues were undertaken. The results clearly demonstrate that the Novikoff hepatoma and the more slowly growing Morris hepatoma #5123 have a considerably lower concentration of coenzyme Q than normal liver, while in regenerating liver the coenzyme Q concentration is comparable to that of the normal liver.

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TABLE I. Tissue Concentration of Coenzyme Q.

Type of tissue	Determinations	CoQ concentration*
Normal liver	8	611 ± 144
48 hr sham-operated control	5	765 ± 166
72 hr ” ” ”	3	658 ± 173
48 hr regenerating liver	5	705 ± 69
72 hr ” ”	3	602 ± 70
Morris hepatoma	6	211 ± 53
Novikoff ”	4	325 ± 87
Heart	2	958 ± 31
Kidney	2	419 ± 85
Spleen	2	212 ± 12

* μg/g dry wt as CoQ$_{10}$ (mean ± stand. dev.).

Materials and methods. Quantitative determination of coenzyme Q was carried out by a modification of the method of Crane *et al.*(7). Weighed amounts of tissue were homogenized in saline and refluxed for 30 minutes in an equal volume of 15% KOH in methanol containing pyrogallol. Non-saponifiable lipids were extracted with isooctane. The isooctane extracts were washed, then purified on a 2 × 3 cm Decalso (50/80 mesh) chromatographic column. The coenzyme Q fraction, eluted with 8% (v/v) ethyl ether in isooctane, was evaporated to dryness *in vacuo* and dissolved in absolute ethanol. Coenzyme Q was determined by measuring optical density at 275 mμ before and after reduction with KBH$_4$. Total coenzyme Q was determined as coenzyme Q$_{10}$, utilizing $\Delta E^{1\%}_{1\,\text{cm}}$ (oxidized-reduced) 275 mμ = 142. Quantitative histochemical procedures for succinate-INT reductase are the same as those used previously(4). Female Sprague-Dawley rats 3-4 months of age obtained from the Holtzman Co. were used throughout, except for work on the Morris Hepatoma 5123.† In hepatic regeneration experiments approximately two-thirds of the liver was excised (8). The Novikoff hepatoma was grown intraperitoneally as a solid tumor(9). Tumors 5 or 6 days following transplantation were used.

Results. The results are summarized in Tables I and II. The coenzyme Q concentration of both the Morris hepatoma and the Novikoff hepatoma is considerably lower than that of normal liver, and in these tissues addition of coenzyme Q$_{10}$ to the succinate-INT reductase reaction results in considerably greater enhancement of activity than is found with the normal liver. Regenerating liver has a coenzyme Q concentration similar to that of normal liver, and enhancement of succinate-INT reductase activity by addition of coenzyme Q is also of a similar order of magnitude to that of normal liver.

Heart, kidney, and spleen from normal rats are included in the present study to show a range of succinate-INT reductase activities and coenzyme Q concentrations in several normal tissues. It will be noted that spleen bears a considerable resemblance to the 2 hepatomas studied in that succinate-INT reductase activity is low, addition of coenzyme Q$_{10}$ causes a large enhancement in this activity, and coenzyme Q concentration of the tissue is of the same order of magnitude as that of the 2 malignant tissues.

Discussion. These results have demonstrated that there is no significant change

TABLE II. Effect of Coenzyme Q$_{10}$ Addition on Succinate-INT Reductase Activity.

	Succinate-INT reductase activity†		
Type of tissue*	No addition	CoQ$_{10}$ added	% increase enzyme activity
Normal liver (2)	179 ± 9	235 ± 7	31
48 hr sham-operated control (3)	198 ± 14	288 ± 25	45
72 hr sham-operated control (3)	177 ± 26	228 ± 38	33
48 hr regenerating liver (3)	96 ± 13	143 ± 24	48
74 hr regenerating liver (3)	147 ± 30	191 ± 25	30
Morris hepatoma (4)	118 ± 12	239 ± 24	103
Novikoff hepatoma (2)	29 ± 1	65 ± .4	117
Heart (2)	362 ± 4	392 ± 39	8
Kidney (2)	297 ± 6	341 ± 13	15
Spleen (2)	69 ± .1	169 ± 7	145

* No. of determinations in parentheses.

† 1000 × ΔO.D. at 490 mμ/mg dry wt/min. (mean ± stand. dev.).

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† The Morris Hepatoma was grown in Buffalo strain rats. We wish to thank Dr. Harold P. Morris, Nat. Cancer Inst., for supplying us with tumor bearing animals.

in tissue concentration of coenzyme Q during liver regeneration, and suggest that relatively rapid coenzyme synthesis takes place in this tissue. High levels of coenzyme are maintained at a time when the cell is under the stringent metabolic demands occasioned by its own reproduction. The low reductase activity can be accounted for by a lack of primary dehydrogenase or by an inability of newly forming dehydrogenase to couple with the adequate amounts of coenzyme Q present. In support of this latter possibility is the observation that menadione causes a relatively greater degree of enhancement of succinate-INT reductase activity in regenerating liver than in normal liver(5).

There is additional support for the concept that endogenous coenzyme Q does not function optimally in the succinate-INT reduction system of sections of regenerating liver. It has been shown(6) that sections of liver from normal rats injected with coenzyme Q_{10} show an enhanced reductase activity and a marked decrease in ability of additional coenzyme Q_{10} to increase the succinate-INT reductase activity when subsequently added *in vitro*(6). These observations indicate that the degree of unsaturation of the succinate-INT reductase system can be altered by changes in amount of coenzyme Q_{10} available to liver cells *in vivo*. In the present study it has been found that the amount of coenzyme Q relative to the succinate-INT reductase activity is considerably higher in regenerating liver than in normal liver. Therefore, it would be expected that the degree of enhancement of reductase activity obtained by addition of coenzyme Q_{10} would be less in regenerating liver than in normal liver. That this is not the case suggests that in regenerating liver intracellular sequestration of the coenzyme Q takes place.

In both the Morris hepatoma and the Novikoff hepatoma a low concentration of coenzyme Q has been found. Both hepatomas likewise have succinate-INT reductase systems whose activities can be markedly enhanced by addition of coenzyme Q_{10}. These findings suggest that in the 2 neoplasms a non-availability of optimal amounts of coenzyme Q is an important factor in the low succinate-INT reductase activities observed. In the Novikoff hepatoma a second factor appears to participate. In this tumor a high ratio of coenzyme Q to succinate-INT reductase activity occurs suggesting that the limited amount of coenzyme Q present does not maximally function in the reductase reaction. Thus there would appear to be either a deficiency in primary dehydrogenase or an inability of the primary dehydrogenase present to couple with the available coenzyme Q. During the 6 months preceding this study a striking decrease, almost 50%, in succinate-INT reductase activity of the Novikoff hepatoma was observed. Ability of coenzyme Q_{10} to enhance the reductase activity also diminished. This tumor tends to become necrotic quite rapidly and while morphologically non-necrotic portions were used for the analytic studies reported it is possible that some of the changes observed are due to inclusion of cells which were undergoing incipient degenerative changes.

The studies of normal tissues have shown that there is a similarity between concentration of coenzyme Q in spleen and the 2 hepatomas and, in addition, that the succinate-INT reductase activity of spleen is as markedly enhanced by *in vitro* addition of coenzyme Q_{10} as is that of the hepatomas. These data provide evidence that the hepatomas do not have an actual deficiency of quinone. Whether or not the pattern of low coenzyme Q concentration and marked unsaturation of the succinate-INT reductase system with respect to quinone has some functional significance remains to be determined.

Conclusion. Quantitative studies of normal and regenerating liver have shown that high concentrations of coenzyme Q are maintained during the proliferative process and indicate that a lack of coenzyme is not responsible for the low succinate-INT reductase activity observed during regeneration. In contrast, a low coenzyme Q concentration in the Morris hepatoma and the Novikoff hepatoma appears to play an important role in the low reductase activities observed in these 2 neoplasms.

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Induction by Sendai Virus of Non-transmissible Cytopathic Changes Associated with Rapid and Marked Production of Interferon.* (26921)

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During experiments with various myxoviruses it was noted that high concentrations of chick adapted Sendai virus induced non-transmissible cytopathic effects (NTCE) in cultures of primary human amnion (HA) cells. Several observations suggested that Sendai virus underwent an incomplete cycle of multiplication in these cells, which was associated with the early liberation of large quantities of an interferon-like substance into the culture medium. These observations are described together with experiments suggesting that these phenomena may be interdependent.

Methods and materials. Cell culture. Preparation and maintenance of human amnion (HA) cells has been previously described (1). Primary cultures of rhesus monkey kidney cells were obtained commercially.[†]

Viruses. Relevant data on viruses employed are as follows:

Sendai virus (F. Davenport) allantois of embryonated egg 27
Sindbis virus (R. Taylor Ar-339) HA cells 10
Poliovirus II MEF chick embryo cell cultures 143, HA cells 2
Mumps virus HA cells 7
Simian virus 5, monkey kidney cells 4
DA virus[‡] (P. Isaacson Rh 76) HA cells 3

Preparation of stock sendai virus. Sendai virus was prepared in 9-11 day embryonated eggs by inoculation intrallantoically of 0.1 ml of a 10^{-3} dilution of stock virus. After 72 hours incubation at 35-37°C, allantoic fluid was harvested, centrifuged for 10 minutes at 400 g, and the supernate centrifuged at 4°C for 2 hours at 110,000 g (Spinco L preparative centrifuge #40 rotor). The sediment was resuspended in a mixture of 50% tissue culture medium and 50% skimmed milk and stored at –70°C.

Virus assay. Infectivity titers were calculated by the formula of Reed and Muench. Sendai virus was titrated in embryonated eggs (EID_{50}) taking as criterion of infection appearance of hemagglutinin in allantoic fluids. Sindbis, poliovirus, DA, SV 5 and mumps viruses were prepared and titrated in HA cells. In this system more than 25% cell destruction was considered evidence of viral multiplication or NTCE in the case of Sendai virus. Hemagglutination and hemadsorption tests were performed by standard methods(2,3).

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** Postdoctoral Research Fellow of National Foundation.

[†] Microbiological Associates, Inc., Washington, D. C.

[‡] A myxovirus isolated from the blood of a patient with viral hepatitis. This virus is considered closely related if not identical to SV 5 and SA viruses.

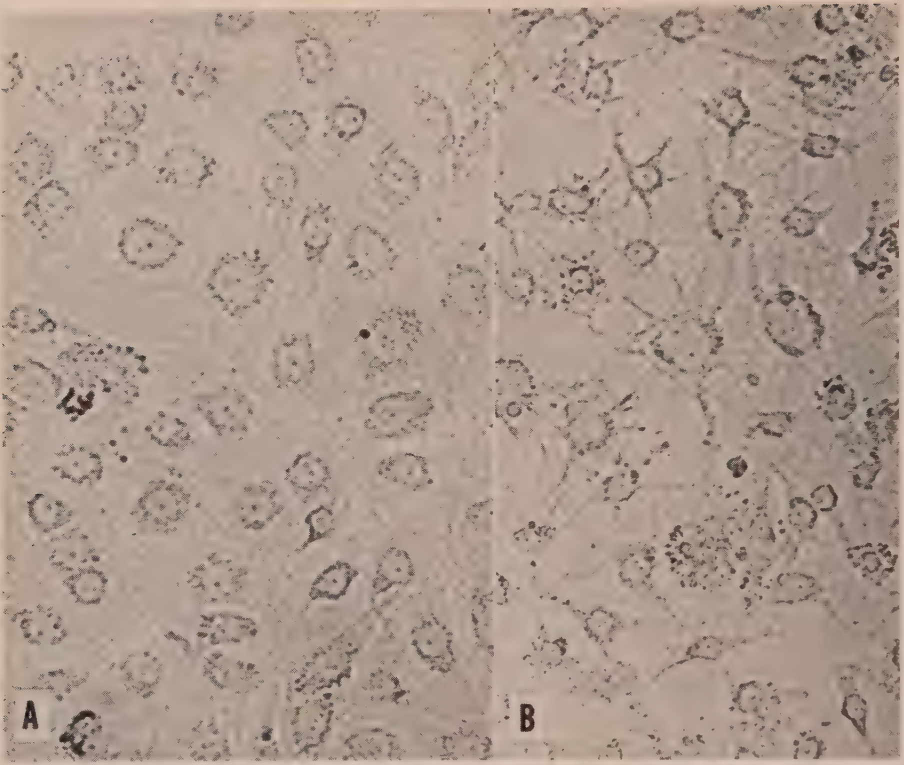

FIG. 1. A. Uninoculated human amnion cells unstained. B. Human amnion cells 24 hr after inoculation of chick-adapted Sendai virus unstained. Mag. × 350.

Antisera. Anti-Sendai rabbit serum was obtained through the courtesy of Dr. J. Lewis, the Sterling Winthrop Co., Rensselaer, N. Y.

Assay of interferon. All preparations of interferon were centrifuged at 110,000 g at 4°C for 2 hours. The supernate (interferon) was free of infectious virus and hemagglutinin, and in some instances anti-Sendai rabbit serum in excess was added. Interferon was assayed in primary tube cultures of HA cells previously incubated in stationary racks at 35-37°C for 4 or more weeks. Two-fold dilutions of interferon (2-3 tubes/dilution) were incubated with cells for 18-24 hours prior to inoculation of 100 $TCID_{50}$ of Sindbis virus. Complete destruction of cells in control cultures regularly occurred in 3 days. Interferon titer was expressed as the highest dilution which protected more than 75% of the cell sheet in at least one of 2 cultures for at least 3 days after complete destruction of cells had occurred in control cultures. Cultures exhibiting more than 25% CPE were regarded as interferon-negative although marked differences from control cultures in time of onset and extent of CPE were often observed.

Results. Morphologic changes. Infected (Sendai virus) allantoic fluid in appropriate dilutions added to cultures of HA cells induced diffuse and marked NTCE. This consisted of shrinkage and loss of cytoplasm, appearance of elongated cytoplasmic process and relative prominence of the nucleus (Fig. 1). NTCE was first noted between 6-12 hours after addition of the agent, depending upon its concentration, and was complete between 24-48 hours. Thereafter affected cells gradually disappeared.

Association of NTCE with infectious virus. These changes were not elicited after inoculation of a) control allantoic fluid, b) ultraviolet irradiated infected allantoic fluid, c) supernate of ultra-centrifuged infected allantoic fluid, or d) aliquots of a previously incubated mixture of Sendai virus and antiserum.

Non-transmissibility of cytopathic effect. From Table I it can be seen that the cyto-

TABLE I. Failure to Transmit Cytopathic Effect Serially in Human Amnion Cells.

Virus dilution	Passage 1	2	3
1:10	+	±	0
1:100	+	0	0
1:1000	±	0	0
1:10,000	0	0	0

+, CPE; ±, partial CPE; 0, absence of CPE.

pathic effect described was not transmissible to uninoculated cultures. At a viral dilution of 1:10 some effect was still noted on second passage. Since on the third passage no changes were observed, it seems evident that this effect in the 2nd passage was due to residual virus derived from the original inoculum. This CPE was not observed on second passage if the original inoculum was removed several hours after exposure to HA cells and the cell sheet washed prior to replacement of maintenance medium.

To induce NTCE in HA cells, large amounts of virus (1-10 EID_{50} per cell) were required, thus bringing the ratio of EID_{50} to TCD_{50} to about 10^{5-6} :1. No multiplication of infectious virus was demonstrated in HA cells after inoculation of large amounts of virus. The presence of 50,000 EID_{50}/ml of virus at 3 hours (Table II) may be attributed to residual virus after removal of the original inoculum and washing of the cell sheet at 1 hour. Forty-eight hours after inoculation, the titer of virus had decreased to 16,000 ID_{50}/ml. With a small inoculum, (*i.e.*, 1000 EID_{50}) of virus, slight multiplication of infectious virus did occur. Thus after removal of the original inoculum and washing of the cell sheet prior to introduction of fresh medium, no infectious virus was detected at 3 hours, and 320 EID_{50}/0.1 ml of virus was present 48 hours after inoculation.

If guinea pig erythrocytes were added just prior to or upon the first indication of NTCE, focal areas of hemadsorption were observed. At the time of maximum NTCE the cell sheet was obscured by adsorbed erythrocytes and hemagglutinin in low concentrations (dilutions of 1:2-1:8) was demonstrable in culture fluid (Table II).

Though other cell virus systems were not extensively investigated, chick-adapted Sendai virus was found to induce NTCE in human kidney, WS and HeLa cells. Monkey kidney cells appeared unaffected by Sendai virus though hemadsorption of guinea pig erythrocytes was demonstrated 2 days after inoculation.

Interferon production. After infection of HA cells with ultra-centrifuged chick-adapted Sendai virus, a substance possessing certain properties characteristic of interferon appeared in the culture medium (Table III). In the viral inoculum itself no interferon was detected. Significant quantities of interferon were found 12 hours after inoculation and maximum titers were attained at 24-48 hours (Table II). The amount of interferon produced was roughly proportional to the concentration of virus in the original inoculum (Table IV). Titers ranging between 1:256 to 1:1024 were consistently recorded.

Species specificity of interferon. High concentrations of Sendai interferon (1:1024) produced in HA cell cultures did not protect chick embryo cells against challenge with 50 $TCID_{50}$ of Sindbis virus.

Interferon prepared in monkey kidney cells

TABLE II. Effects of Sendai Virus on HA Cells.

Time (hr)	1.5*	3	6	12	24	48
CPE	0	0	Trace	50%	100%	100%
Hemadsorption	0	0	Trace	Marked		
Hemagglutinin (uncentrifuged medium)	<1:2	<1:2	<1:2	<2	1:2	1:8
Egg infectious virus log ID_{50}/ml	—	4.7	—	—	—	4.2
Titer of interferon produced (ultracentrifuged supernate)	<1:4	1:6	1:6	1:96	1:768	1:768

* Original inoculum $10^{8.3}$ EID/ml was removed after 1 hr incubation at 37°C and the cell sheet washed several times with Hanks' salt solution before replacement of maintenance medium.

TABLE III. Properties of Interferon Liberated by HA Cells after Infection with Sendai Virus.

Procedure	Result
Incubation with crystalline	
Trypsin, 0.5 mg/ml, 37°C 1 hr	Complete loss
DNA-ase, *idem*	No effect
RNA-ase, "	" "
Heat 60°, 1 hr pH 7.8	Complete loss
20% ether, 18 hr 4°C	4-8 fold decrease
pH 4, dialysis, 24 hr 4°C	No effect
Storage 4°C–20°C, 2 mo	" "
Incubation with Sendai antiserum	" "
Ultracentrifugation 110,000 g, 2 hr 4°C	" "

by infection with Sendai virus was effective at a dilution of 1:512 in protecting HA cells against challenge with 100 $TCID_{50}$ of Sindbis virus, but did not protect chick embryo cells at a dilution of 1:4.

Relative lack of effectiveness of interferon against NTCE. Interferons produced by HA cells after infection with either Sendai or measles virus were effective at dilutions of 1:1024 (Sendai) or 1:256 (measles) in protecting HA cells against challenge with a 100 $TCID_{50}$ of Sindbis or polio viruses. The same interferon preparations, however, were far less effective in preventing the NTCE in HA cells induced by Sendai virus. Thus with interferon dilutions of 1:2 only a 16-fold decrease in NTCE titer was observed, and at interferon dilutions of 1:4 and 1:8 the difference in NTCE between treated and untreated cells was much less (4- and 2-fold respectively).

Human amnion cells were infected with mumps, SV_5 or DA viruses, and challenged with Sendai virus 4-6 days later when cytopathic effect due to these agents was minimal. No NTCE was observed even after introduction of 10^8 EID_{50} of Sendai virus. This protection afforded by previous infection of cells contrasted with the relative ineffectiveness of stock interferon to repress significantly Sendai NTCE. Moreover this effective inhibition could not be attributable to the presence of virus or interferon in the medium nourishing infected cells, since it was undiminished after removal of medium and washing of the cell sheet prior to challenge. When appropriate antisera (anti-mumps, anti-SV5 etc.) were added to cultures previously infected with these agents before challenge with Sendai virus no hemadsorption occurred, though in challenged uninfected control cultures NTCE and hemadsorption were noted. Absence of Sendai NTCE in cultures previously infected with the other agents was therefore confirmed by the absence of hemadsorption. Accordingly one may infer that previous infection of cells with these viruses either prevented entry of Sendai virus into the cell or prevented viral replication and NTCE.

Discussion. The non-transmissible cytopathic effect in HA cells occurring several hours after addition of high concentrations of infectious chick-adapted Sendai virus resembles that observed with other myxoviruses in various cell cultures(4). The yield of hemagglutinin was in most instances low and that found may possibly represent release of hemagglutinin adsorbed from the original inoculum. Even if this were true, however, the appearance of hemadsorption 6 hours after inoculation, concomitant with onset of NTCE and failure to demonstrate an increase in infectious virus strongly suggest an incomplete cycle of viral multiplication. Whether cellular destruction results from cytoplasmic replication of viral components that remain non-infectious or whether it results from a mere accumulation from the inoculum of a large number of infectious viral particles within the cell remains undetermined.

The similarities between the viral inhibitory substance present in the medium of Sendai infected HA cells and previously described interferons would seem to justify application of the term interferon to this factor. It must be emphasized that significant levels of interferon were attained as early as 12

TABLE IV. Effect of Dilutions of Sendai Virus on Production of Interferon.

Dilution of virus*	CPE (48 hr) %	Interferon titer (48 hr)
10^{-2}	100	1024
10^{-3}	100	384
10^{-4}	100	384
10^{-5}	0	64
10^{-6}	0	12

* Egg infectivity titer 10 $EID_{50}/10^{9.3}$ EID_{50}/0.1 ml.

hours after inoculation. The rapid rate of interferon production and high titers obtained may be idiosyncratic of this virus. On the other hand it is tempting to speculate that the "success" of interferon production is related to the failure of HA cells to support multiplication of infectious virus. Whether the early appearance of interferon is responsible itself for this abortive cycle of multiplication remains speculative. Some support for this latter hypothesis may be found in the demonstration of slight multiplication of infectious virus in HA cells after inoculation of a relatively small concentration of virus.

Henle and coworkers(5) noted that Sendai virus underwent an incomplete cycle of replication in MCN cells. To assay the effect of various interferons on Sendai virus infection in these cells, they determined the titer of hemagglutinin liberated after challenging control cultures and cultures pretreated with undiluted interferon. A decrease in hemagglutinin titer of 8-100 times was observed after preincubation of cells with interferon for 24 hours. These results are comparable to the 16-fold decrease in NTCE titer observed in our experiments after incubation of HA cells with undiluted or very low dilutions of interferon. This same interferon preparation, however, when diluted several hundred fold completely inhibited CPE after inoculation of over a 100 $TCID_{50}$ of Sindbis or polio virus. If Sendai NTCE can eventually be shown to depend upon the cytoplasmic incomplete cycle of viral synthesis our results would afford support for Isaac's contention that interferon is less effective in suppressing viral components in the cytoplasm than it is in the nucleus(6).

To follow this line of speculation further it is possible that interferon may play a decisive role in the well-known increase of the hemagglutinin/infective virus ratio that follows inoculation of high concentrations of certain viruses (*i.e.*, influenza virus). Thus in serially passing high concentrations of influenza virus, one may well also transfer interferon. If cytoplasmic viral reproduction is less affected by interferon, the production of hemagglutininative virus would be less inhibited by interferon than that of complete virus.

It may be pointed out that the contrast between the marked resistance of cells previously infected with other viruses to NTCE of Sendai virus and that conferred by interferon indicates that the mechanisms of the resistance may well be different.

Summary. High concentrations of chick-adapted Sendai virus induced non-transmissible cytopathic effects in cultures of primary human amnion (HA) cells. Evidence was presented suggesting that an incomplete cycle of Sendai virus multiplication occurred. An interferon-like substance was liberated as early as 12 hours after inoculation of this virus. Titers of interferon ranged between 1:256 and 1:1024 as assayed in HA cells challenged with Sindbis or poliovirus. This and other interferons were far less effective in protecting HA cells against the non-transmissible cytopathic effect of Sendai virus. In contrast, however, these cells when previously infected with mumps, SV_5, and DA viruses proved completely resistant to challenge with high concentrations of Sendai virus.

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Effect of Combined Use of Calcium Gluconate and Ammonium Chloride on Removal of Sr^{85} in Man Two Weeks after Exposure.[*] (26922)

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Studies of the metabolism of radioactive strontium in man(1,2) and of the enhancement of its excretion have been previously reported from this laboratory(3-5). An increase of the urinary calcium excretion induced by intravenously administered calcium and/or orally administered ammonium chloride was shown to be accompanied by a considerable increase of urinary radiostrontium excretion(1,3,5). The possibility of inducing an increase of radiostrontium excretion by orally administered ammonium chloride as late as 2 weeks after injection of the Sr^{85} tracer has been demonstrated(3). This relatively late removal is of interest since radiostrontium is assumed to be mainly deposited in tissues, including bone, at that time. Since the combination of intravenously injected calcium and orally administered ammonium chloride was more effective than either agent alone at the time of injection of the Sr^{85} tracer, in the so-called "early" phase of the study, the effectiveness of this combined regimen 2 weeks after exposure was investigated. These data are reported here.

Materials and methods. Six patients were studied on a constant, analyzed, dietary intake under strictly controlled conditions on the Metabolic Research Ward. Duration of the studies ranged from 19 to 28 days. The age, sex and diagnoses of the patients and the studies performed are listed in Table I.

A single tracer dose of Sr^{85} (0.1-0.2 μc/kg body weight) was given intravenously on the first day of the control and experimental studies. Plasma levels of Sr^{85} were determined at 1, 4, 8 and 24 hours following injection of the tracer, daily for 6 days thereafter and 3 times per week for as long as significant counts could be obtained with the equipment available. The urinary output of the first day of the study was collected in fractions, for radioassays and calcium analyses, at time intervals at which blood samples were obtained. Thereafter, urinary Sr^{85} and calcium excretions were determined daily on aliquots of 24-hour urine collections. Each stool specimen was assayed for Sr^{85} during the entire study. Starting on the 13th day following intravenous injection of Sr^{85}, 9 g ammonium chloride were given orally per day on 5 consecutive days. Infusions of 500 ml of 5% glucose in water containing 50 ml calcium gluconate (469 mg Ca^{++}) were given daily for a 4-hour period on 3 successive days (study days 15, 16 and 17) so that ammonium chloride and infusions of calcium were given simultaneously for 3 days.

The technic of administration of Sr^{85} and of assaying Sr^{85} plasma levels and urinary and fecal excretions has been previously described(2,3).

Metabolic balances of nitrogen, calcium and phosphorus were determined on aliquots of 6-day pools of urine and stool collections and on aliquots of the diet during the control and experimental studies to ascertain that the metabolic status of the patients remained unchanged at the time the various studies were performed. Nitrogen balances were not determined during the period of ammonium chloride administration.

The diet and excreta were analyzed for nitrogen, calcium and phosphorus on aliquots of each 6-day metabolic pool. Analyses of urinary and fecal nitrogen were performed by the Kjeldahl method, of phosphorus by the method of Fiske and SubbaRow(6) and of urinary calcium by the method of Shohl and Pedley(7). Stool calcium and phosphorus were determined on ashed aliquots of 6-day collection pools.

Results. Fig. 1 illustrates the comparative effect of intravenously administered calcium

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TABLE I. List of Patients Studied.

Patient	Age	Sex	Diagnosis	Studies performed*
1	71	♂	Mild osteoarthritis	Intrav. calcium
2	52	♀	Carcinoma of breast	" "
3	63	♀	Osteoporosis	" " " " and oral ammonium chloride
4	77	♀	"	Intrav. calcium " " and oral ammonium chloride
5	73	♂	Carcinoma of prostate	Intrav. calcium " " and oral ammonium chloride
6	70	♀	Osteoporosis	Intrav. calcium " " and oral ammonium chloride

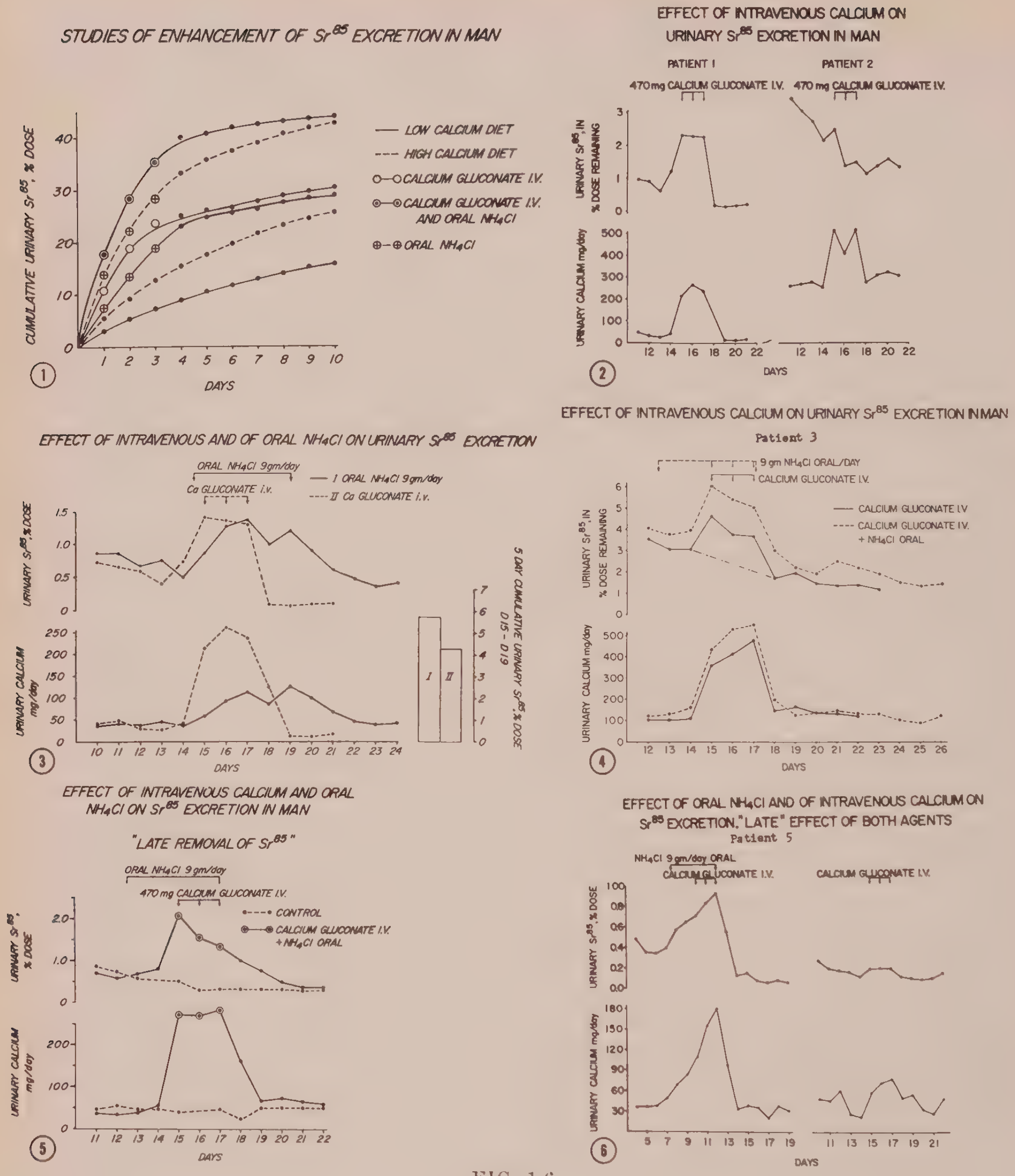
* A tracer dose of Sr^{85} was given intrav. on first day of each study. Intrav. calcium was given for 3 days, starting on study day 15. In studies in which the combination of intrav. calcium and oral ammonium chloride was used, 9 g NH_4Cl were given orally per day for 5 days starting on study day 13.

and of orally administered ammonium chloride on urinary Sr^{85} excretion at the time of Sr^{85} injection. Calcium and ammonium chloride were given singly and in combination in different studies to Patient 1. Urinary Sr^{85} excretion was lowest during low calcium intake. The enhancement of urinary Sr^{85} excretion obtained with intravenously infused calcium or orally administered ammonium chloride was of similar magnitude. A combination of both agents resulted in the greatest increase in Sr^{85} excretion. For comparative purposes, the effect of high calcium intake alone and that of ammonium chloride during high calcium intake on Sr^{85} excretion are also graphed in Fig. 1. During high calcium intake, urinary Sr^{85} excretion was almost as high as during the oral administration of ammonium chloride during low calcium intake. Ammonium chloride, given orally during high calcium intake, resulted in a 10-day cumulative urinary Sr^{85} excretion of similar magnitude to that obtained by the combined use of intravenously administered calcium and orally administered ammonium chloride during low calcium intake.

Fig. 2 shows the effect of infusions of calcium gluconate on urinary Sr^{85} excretion on the 15th, 16th and 17th day following injection of Sr^{85} in 2 patients who were in different states of calcium metabolism. In Patient 1, each calcium infusion considerably increased Sr^{85} excretion. In contrast, the urinary excretion of Sr^{85} of Patient 2 showed irregular fluctuations on the days of calcium infusion and only a slight, if any, increase occurred. Before calcium gluconate was infused, the urinary Sr^{85} excretion of Patient 2 was approximately 3 times and urinary calcium excretion approximately 10 times as high as that of Patient 1. The calciuria of both patients increased on the 3 days of calcium infusion and returned to approximate pre-infusion levels on subsequent days.

Fig. 3 shows the comparative effectiveness of intravenously injected calcium and of orally administered ammonium chloride 2 weeks after injection of the Sr^{85} tracer (Patient 1). The prompt rise of urinary Sr^{85} excretion observed when calcium was infused contrasts with the gradual increase of urinary Sr^{85} excretion when ammonium chloride was given orally for several days. Increased Sr^{85} excretion persisted for 2 to 3 days after discontinuation of ammonium chloride. The 5-day cumulative urinary Sr^{85} excretion (days 15-19) was higher when ammonium chloride was given than following calcium gluconate infusions. The changes in urinary calcium in both studies are shown in the lower half of this graph.

Fig. 4 presents the results obtained with combined use of intravenously infused calcium and orally administered ammonium chloride 2 weeks after injection of the Sr^{85} tracer (Patient 3). A distinct increase in urinary Sr^{85} excretion occurred when calcium gluconate was infused on study days 15, 16



STUDIES OF ENHANCEMENT OF Sr^{85} EXCRETION IN MAN

EFFECT OF INTRAVENOUS CALCIUM ON URINARY Sr^{85} EXCRETION IN MAN

EFFECT OF INTRAVENOUS AND OF ORAL NH_4Cl ON URINARY Sr^{85} EXCRETION

EFFECT OF INTRAVENOUS CALCIUM ON URINARY Sr^{85} EXCRETION IN MAN Patient 3

EFFECT OF INTRAVENOUS CALCIUM AND ORAL NH_4Cl ON Sr^{85} EXCRETION IN MAN "LATE REMOVAL OF Sr^{85}"

EFFECT OF ORAL NH_4Cl AND OF INTRAVENOUS CALCIUM ON Sr^{85} EXCRETION. "LATE" EFFECT OF BOTH AGENTS Patient 5

FIG. 1-6.

and 17 (solid line) and urinary Sr^{85} excretion was further increased when ammonium chloride was given simultaneously (dotted line). Before calcium and ammonium chloride were administered, 65.6% of the originally administered dose of Sr^{85} had been excreted. Sr^{85} excretion is therefore expressed in per cent of the administered dose remaining in the body at the time the enhancing agents were administered.

Fig. 5 shows a distinct rise of urinary Sr^{85} excretion when ammonium chloride was given orally and calcium gluconate intravenously to Patient 6. This graph also shows that the elevation of the urinary Sr^{85} excretion persisted for 2-3 days after discontinuation of ammonium chloride.

The contrasting effects obtained in Patient

TABLE II. Effect of Oral NH_4Cl and of Intravenous Calcium on Sr^{85} Excretion in Man (Late Phase).

	Sr^{85}, % dose remaining on day 14		Urinary Sr^{85} excretion, days 15-20, % of dose remaining			Factor of increase (study phase)	
Patient	Control 1	Control 2	Control	Ca gluc.	Ca gluc. + NH_4Cl	Ca gluc.	Ca gluc. + NH_4Cl
1	61.6	—	1.6	7.0	—	4.4	—
2	23.9	—	7.1	7.5	—	none	—
3	34.4	31.7	9.3	14.2	21.1	1.4	2.3
4	41.3	44.6	5.8	11.9	15.0	2.1	2.6
5	90.4	92.3*	.6, 1.0*	.8	4.0*	none	4.0
6	55.4	54.3	5.7	7.5	16.5	1.3	2.9

* Days 8-12 inclusive.

Control 1 = prior to infusion of calcium gluconate.
Control 2 = prior to combined administration of oral NH_4Cl and of intrav. calcium.

5 with calcium infusions and with the combination of intravenously infused calcium and orally administered ammonium chloride are illustrated in Fig. 6. The urinary Sr^{85} and calcium excretions were extremely low in this patient before either agent was given. When calcium gluconate was infused on study days 15, 16 and 17, there was only a very slight and irregular increase of urinary Sr^{85} excretion. However, the combined use of intravenous calcium and oral ammonium chloride led to an approximate 3.5-fold increase of urinary Sr^{85} excretion, which was accompanied by an approximate 6-fold increase of urinary calcium.

Table II shows that the combined use of intravenously injected calcium and orally administered ammonium chloride was more effective in increasing Sr^{85} excretion than infusions of calcium alone. This is best illustrated by the data of Patient 3. There was little, if any, increase in Sr^{85} excretion on the days of the calcium infusions but the use of intravenous calcium together with oral ammonium chloride increased urinary Sr^{85} excretion approximately 4-fold.

Discussion. Very few agents are available for the effective removal of radiostrontium. Changing the calcium/phosphorus ratio of the diet, for instance, either by increasing calcium intake or by drastically decreasing dietary phosphorus, has been shown to reduce the skeletal radiostrontium content in experimental animals(8,9). However, this severe reduction of phosphorus intake led to osteomalacia. Also, this procedure is impractical in man, since severe restriction of phosphorus intake can only be achieved by simultaneous reduction of dietary protein intake. The effect of increased calcium intake has been studied in man and was found to be of limited value in decreasing the absorption of Sr^{85} and increasing its excretion(10,11). Chelating agents are only of limited value in the removal of radiostrontium because of the higher stability constants for calcium than for strontium(12). The use of sodium salts of chelating agents may, in fact, lead to a decrease in radiostrontium excretion(13).

Extensive studies of the removal of radiostrontium from man have been carried out in this laboratory in the past few years. The increase in excretion obtained with ammonium chloride is in agreement with some of the data reported by Hamilton on removal of radiostrontium from rats(14). The absolute amounts of radiostrontium removed from man by ammonium chloride 2 weeks after exposure were small but one has to consider that as much as 50% or more of the administered dose had been excreted by the time the enhancing agents were administered. It is not clear whether the increased radiostrontium excretion in the "late" phase is due to removal of radiostrontium from bone or from soft tissue. It is usually assumed that the major fraction of the retained radiostrontium is deposited in bone in 2 weeks, so that a negligible fraction of the administered dose would be present in soft tissue at that time. Jaffe *et al.* have shown that demineralization of the skeleton can be induced in dogs by the use of ammonium chloride(15). The increase in urinary calcium during administration of ammonium chloride in the studies reported here indicates that calcium was removed from bone due to acidosis and Sr^{85} may, therefore, also have been concomitantly removed from the skeleton. However, the induced excess of Sr^{85} excretion in man may be in part due to the removal of Sr^{85} from soft tissue rather than from bone, since tissue distribution studies carried out in man with tracer doses of Sr^{85} and Ca^{45} have shown that the soft tissue concentration of Sr^{85} was higher than expected several weeks after injection of the tracer(16).

The studies reported here indicate that the use of ammonium chloride together with intravenously administered calcium is more effective than the use of either agent alone 2 weeks after injection of the tracer. The calcium metabolism of the individual and the percentage of radiostrontium retained in the body at the time of the use of the enhancing agents are decisive factors in increasing Sr^{85} excretion. This is well-illustrated by the contrasting effect obtained with infused calcium in Patients 1 and 2. Although increased calciuria was observed in both patients, the urinary Sr^{85} excretion did not increase in Patient 2 who had both a very high urinary calcium excretion and a high Sr^{85} excretion prior to the calcium infusion. The small percentage of the dose of Sr^{85} which remained in the body at that time may have been inaccessible for removal. In studies in which other agents were used, the increase of Sr^{85} excretion was minimal in persons who had a high urinary calcium and therefore, frequently, also a high urinary radiostrontium excretion(4,5). Similarly, the state of calcium metabolism also played an important role in the failure to increase Sr^{85} excretion by intravenously administered calcium in Patient 5, who had osteoblastic bone metastases and a great tendency to retain calcium. However, the simultaneous use of orally administered ammonium chloride apparently led to active demineralization and to a relatively high excretion of calcium which was accompanied by increased radiostrontium excretion.

Summary. 1. Studies of the removal of radiostrontium (Sr^{85}) from man have been carried out under controlled dietary conditions with the combination of orally administered ammonium chloride and intravenously infused calcium. 2. Each agent alone increased Sr^{85} excretion 2 weeks after administration of the tracer. 3. The use of both agents in combination was more effective in removal of radiostrontium than either agent alone. 4. The effectiveness of either modality of treatment greatly depended on the calcium metabolism of the individual.

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Influence of Dietary Factors on Plasma Lipid Relationships in the Growing Chick. (26923)

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The relationship of serum phospholipid to cholesterol has received considerable attention. Data have been presented(1) demonstrating an increasing cholesterol-phospholipid (C/P) ratio in patients suffering from conditions known to be predisposing to atherosclerosis. The cholesterol-fed chicken(2,3) or rabbit(4,5) and the cholesterol-fed, thiouracil-treated dog(6) all have been shown to have higher C/P ratios than comparable controls.

The level of glyceride in serum has received little attention. Some evidence has been presented indicating that serum glyceride level may be related to atherogenesis(7).

This report presents data on the plasma lipid components of chicks fed differing levels of protein and cholesterol; the data presented illustrate differences in plasma lipid relationships in normal and cholesterol-fed chicks.

Methods. Male Hy-line chicks were used in these studies. The animals were housed in electrically heated cages with raised wire floors, were fed a commercial diet for one week, then distributed to the various treatment groups on the basis of body weight. The dietary variables introduced are indicated in the table of results and the diets were in all other respects adequate.

Body weights and food consumption were determined weekly. Food and water were supplied *ad libitum.* At the end of the experimental period, blood was drawn by cardiac puncture, heparin being used as an anticoagulant.

The collected plasma was extracted with 2:1 chloroform-methanol. The extract was evaporated to dryness under a stream of nitrogen and redissolved in hexane. The lipids were then separated by silicic acid chromatography(8). The eluted fractions were quantitated by the following methods: glycerides by the method of Van Handel and Zilversmit(9) as modified by Leveille *et al.*(10), cholesterol by the method of Searcy and Bergquist(11) and phospholipids by the method of Fiske and Subbarow(12).

Excreta were collected and homogenized; an aliquot of the freeze-dried homogenate was used for bile acid determination by the method of Mosbach *et al.*(13).

Results and discussion. The influence of dietary protein and cholesterol on weight gain, serum lipids and bile acid excretion is presented in Table I. The data demonstrate an increase in all plasma lipids at the lower protein level fed. The observed increase in plasma cholesterol is in accord with the results of previous studies(14,15,16). The increased bile acid excretion noted for the chicks fed the high protein diet is in agreement with the findings of Nishida *et al.*(17), who have shown that chicks fed a high protein diet excrete labeled cholesterol in the bile at a faster rate than do chicks fed a low level of dietary protein. The increase in plasma lipids due to cholesterol feeding is evident from the data in Table I.

The relationship between plasma lipids was studied with the data presented in Table I and similar data obtained in other studies. The correlation coefficient for total plasma cholesterol and phospholipids, in chicks fed a cholesterol-free diet, was found to be highly significant ($r = 0.90$, $P < 0.01$) (Fig. 1). Ester and free cholesterol levels were also significantly correlated to the plasma phospholipid level ($r = 0.90$ and 0.77, respectively). If the C/P ratio is calculated from any point on the regression line shown in Fig. 1, the ratio remains constant, indicating that cholesterol and phospholipid levels increase at the same rate in the chick fed an essentially cholesterol-free diet. If the data for cholesterol-fed chicks are similarly treated, a significant correlation ($r = 0.62$, $P<0.02$) between plasma cholesterol and phospholipid levels results. However, in contrast to the data for chicks fed cholesterol-free diets, calculation of C/P ratios from the regression line (y =

TABLE I. Influence of Dietary Protein and Cholesterol on Weight Gain, Plasma Lipids and Bile Acid Excretion in the Growing Chick.

			Plasma lipids					Bile acid excretion. g/kg body wt/day‡
			Cholesterol, mg/100 ml					
Dietary* protein level, %	Dietary cholesterol, %	Body wt gain, g†	Ester	Free	Total	Glycerides, mM/l	Lipid phosphorus, mg/100 ml	
10	—	89 ± 22§	107 ± 18§	24 ± 7§	131 ± 23§	1.48 ± .63§	9.46 ± 2.49§	3.06
10	.30	84 ± 34	378 ± 86	146 ± 61	523 ± 144	3.59 ± 1.06	12.60 ± 1.63	4.71
20	—	228 ± 18	74 ± 18	27 ± 8	102 ± 20	.41 ± .19	6.41 ± 1.94	4.45
20	2.00	245 ± 21	340 ± 61	169 ± 44	509 ± 104	.95 ± .38	11.30 ± 2.63	5.49

* Sesame oil meal supplemented with 0.90% L-lysine HCl served as the protein source.

† Wt gain from 7th to 28th day; animals fed a commercial diet for days 1-6.

‡ Sum of cholic and deoxycholic acid for one 24-hr collection.

§ Mean of 8 animals ± stand. dev.

149.73 + 0.287x, where y = phospholipid level in mg/100 ml and x = total cholesterol in mg/100 ml) results in increasing ratios at the higher plasma cholesterol levels. These results demonstrate a significant difference in the relationship of these 2 lipid classes to each other in normal and cholesterol-fed chicks. It would appear from these data, that in the chick fed a cholesterol-free diet, the increase in plasma cholesterol is endogenous in nature and is accompanied by a corresponding increase in phospholipids. In the cholesterol-fed chick, the exogenous cholesterol is reflected in elevated plasma levels; and although plasma phospholipids increase in an apparent attempt to maintain a normal relationship, such is not attained and an abnormal ratio results. The significance of the C/P ratio in atherogenesis is at this time speculative; however, the possible importance of an abnormal ratio should be considered.

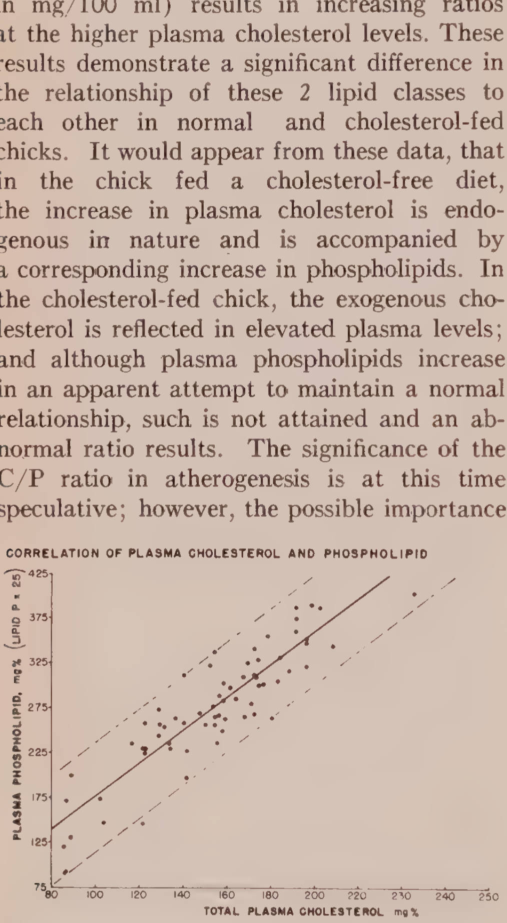

FIG. 1. Relationship of total plasma cholesterol and phospholipid levels in the growing chick. Upper and lower lines represent the 5% confidence limits calculated for the regression line (y = 1.86x − 9.26, where y = plasma phospholipids and x = total plasma cholesterol in mg/100 ml).

The chicks fed cholesterol had higher plasma glyceride levels (Table I) than did chicks fed a cholesterol-free diet; however, no correlation was found between the plasma glycerides and total cholesterol (r = 0.004) or phospholipids (r = −0.011) in the chick not receiving added cholesterol in its diet.

Summary. Chicks fed 20% dietary protein had lower plasma total cholesterol, phospholipid and glyceride levels than did chicks fed a 10% level of protein. The chicks fed the higher level of protein excreted 4.45 g of bile acids per kilo of body weight per day as compared to 3.06 g for the chicks fed the 10% level of protein. A highly significant positive correlation was observed between plasma total cholesterol and phospholipid levels in chicks fed cholesterol-free diets. The data illustrate that cholesterol and phospholipid increase at similar rates in animals not fed cholesterol, but that in the cholesterol-fed chick cholesterol increases at a faster rate, thereby resulting in an elevated cholesterol-phospholipid ratio.

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Swelling of Heart and Liver Mitochondria from Magnesium Deficient Rats and Its Reversal.* (26924)

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Heart mitochondria isolated from rats fed a magnesium deficient diet have a low P/O ratio(1). The uncoupling effect of thyroxine on oxidative phosphorylation is reversed by dietary magnesium(2). However, it is not clear whether the effect of thyroxine and magnesium is related to the biochemical or to the physical integrity of the mitochondrial system(3).

This paper presents a study of the swelling of mitochondria of the heart and liver of rats fed a magnesium deficient diet. Of particular interest is the observation that these mitochondria rapidly became swollen, although their magnesium content was not significantly decreased. ATP† reversed the swelling of mitochondria isolated from the heart and liver of both control and magnesium deficient rats. EDTA prevented the swelling of mitochondria isolated from liver(4,5), but did not prevent the swelling of heart mitochondria, which contained almost twice the amount of magnesium found in liver mitochondrial fraction.

Methods. Young albino male rats, weighing 45 to 50 g were fed diets containing the following constituents per 100 g of diet: casein (purified) 20; sucrose 30; wheat starch 34; salt-mixture of Jones Foster(6) with $CaCO_3$ and $MgSO_4$ removed, 3.0; vitamins A, D and E mixture 1.0; soybean oil 10.0; and $CaCO_3$ 2.0. The vitamins A, D and E mixture contained 5 g of halibut liver oil, 1 g of α-tocopherol acetate and 34 g of soybean oil. The amounts of the water-soluble vitamins used in the diet have been listed elsewhere(7). Magnesium was added to the control diet in the form of MgO to supply 48 mg of magnesium per 100 g of diet. The magnesium deficient diet contained less than 2 mg % of magnesium.

The rats were housed in individual cages, and fed *ad libitum*. The temperature was maintained at 20 ± 2°C. The rats were weighed twice weekly and observed daily for

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* Supported in part by grants-in-aid from Rockefeller Foundation, New York, Japanese Association of Life Insurance Companies, Nat. Heart Inst. and Fund for Research and Teaching, Department of Nutrition, Harvard.

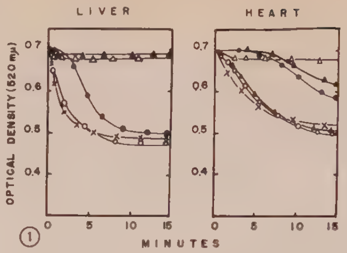
† Abbreviations: ATP, adenosine triphosphate; EDTA, ethylene-diaminetetraacetic acid; Tris, tris (hydroxymethyl) aminomethane; DPN, diphosphopyridine nucleotide.

10 to 12 days for signs of magnesium deficiency. At the end of this period, the rats were sacrificed.

Liver and heart mitochondria were isolated by Lehninger's method(5). The swelling of heart and liver mitochondria was measured spectrophotometrically(5). The standard system was 3.0 ml of 0.25 M sucrose, 0.02 M tris buffer, pH 7.4. An Hitachi spectrophotometer (equipped with thermospacers to maintain temperature in the cuvette carriage chamber at 37°C) was used unless otherwise specified.

The swelling rate of mitochondria was expressed as the reciprocal of the time required to reach one-fourth the maximal extent of swelling as determined by absorbency (called "a reciprocal of quarter time," $\frac{1}{t_{1/4}}$ min.$^{-1}$(5)). Precautions were taken to keep the temperature of the reaction medium constant in order to investigate the difference in rate of swelling between contral rats and magnesium deficient rats. Before adding the isolated mitochondrial suspension kept at 0°, the reaction medium in a Beckman cell was kept in a constant temperature water bath, and the cuvette carriage chamber was kept at a constant temperature by circulating the warm water.

Magnesium and phosphorus concentrations of isolated mitochondria were determined in animals other than those sacrificed for the studies on mitochondrial swelling as follows: The original stock suspension of mitochondria suspended in sucrose solution was centrifuged at 10,000 r.p.m. for 10 minutes. The pellets were homogeneously dispersed in distilled water. One-tenth to 0.25 ml was taken for microkjeldahl analysis of nitrogen. The remainder was deproteinized by addition of 3 volumes of 10% of trichloroacetic acid at 0°C. The supernatant was used for determination of phosphorus and magnesium by methods described elsewhere(8,9). Two rats from each group were used for determination of magnesium and phosphorus in mitochondria, and for determination of the swelling rate of heart mitochondria.



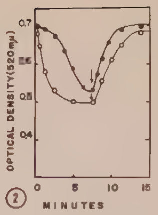


FIG. 1. Rates of swelling of heart and liver mitochondria from rats fed a diet containing 48 mg % magnesium and a diet deficient in magnesium. Measurements were made at 520 mμ, temp. 37°. The following additions were made to 0.25 M sucrose—0.02 M Tris, pH 7.4: ●—● no addition of control rats, ○—○ no addition of magnesium deficient rats; △—△ ATP (0.004 M), ▲—▲ EDTA (0.001 M), ×—× thyroxine (5×10^{-5} M) additions to both of control and magnesium deficient rats. In analysis of liver, 2 rats were used for each point. For heart, one rat was used for each.

FIG. 2. Reversal of mitochondrial swelling in control and magnesium deficient liver by ATP in KCl medium. Measurements were made at the same conditions described in Fig. 1. At zero time: ●—● no addition of control liver mitochondria, ○—○ no addition of magnesium deficient liver mitochondria. At 7.5 min., ATP (0.004 M as a final concentration) was added.

The electron microscopic observations were carried out by the methods described by Palade(10).

The final concentration of L-thyroxine was varied from 1.7×10^{-4} to 5×10^{-5} M.

Results. Liver and heart mitochondria isolated from magnesium deficient rats showed a higher rate of spontaneous swelling than those from controls (Fig. 1, Table I). The rate of spontaneous swelling of heart mitochondria from magnesium deficient rats was much higher at 37° than at 20° (see Exp. No. 3 in Table I).

Effects of thyroxine, ATP, EDTA and DPN on rate of spontaneous swelling. In this experiment thyroxine accelerated the swelling rate of heart mitochondria of the control rat as shown in Fig. 1. The effect of addition of thyroxine was greater in control than in magnesium deficient mitochondria, since thyroxine produced a slight increment or no increment in rate of swelling of mitochondria isolated from magnesium deficient rats. This result was confirmed in the experiment using 0.25 M sucrose, 0.02 M tris buffer medium. The effect of addition of ATP on the swelling rate was determined in

TABLE I. Swelling Rate of Mitochondria $\left(\frac{1}{t_{1/4}\ \text{min.}}\right)$ at 37°C.

	Heart*		Liver†	
Exp.	Control	Mg-def.	Control	Mg-def.
1	.077	.33	.3	.4
2	.10	.33	.4	1.1
3	.12 (.087)	.33 (.087)	.44 (.10)	.60 (.12)
4	.12	.74	.44 (.06)	.82 (.20)
5	.10 (.076)	.20 (.135)	.46 (.24)	.81 (.50)

( ), values at 20°C.

* Two rats were used for each control and each magnesium-deficient determination in each experiment, No. 1 through No. 5.

† One rat was used for each determination in each experiment.

0.15 M KCl, 0.02 M tris medium. ATP completely prevented the spontaneous swelling of liver and heart mitochondria isolated from both the control and the magnesium deficient rats. ATP also completely reversed the swelling after it had nearly reached the terminal absorbency plateau in a medium of 0.15 M KCl, 0.02 M tris buffer (Fig. 2). This effect was confirmed in the control and magnesium deficient mitochondria. EDTA, which was added to investigate the action of metal chelating agent in KCl-tris medium, showed a protective effect against swelling of liver mitochondria of both control and deficient rats (Fig. 1). Similar observations have been noted by others(4,5,11). However, EDTA did not prevent or reverse the spontaneous swelling of heart mitochondria isolated either from control or magnesium deficient rats. Addition of DPN (final concentration of 0.3 mM) produced no demonstrable effect on rate of mitochondrial swelling under these conditions.

Determination of magnesium and phosphorus in mitochondrial fraction. There was a considerable degree of variation in magnesium and inorganic phosphorus content of mitochondria from rat heart and liver. No statistically significant decrease was found in magnesium in mitochondria of both heart and liver obtained from magnesium deficient rats (Table II). Each determination and S.D. was calculated from 5 runs, each using 2 rats. Magnesium and inorganic phosphorus were determined on the same group of rats.

Demonstration of mitochondrial swelling in vivo. Mitochondria in cardiac muscle of magnesium deficient rats occasionally showed a morphological disorganization of fine structure and a swelling *in vivo* (Fig. 3 and 4).

Discussion. Growth inhibition produced in young rats by addition of thyroxine to the diet is partially overcome by extra magnesium supplements. The amount of magnesium required is related to the amount of thyroxine added(2). The effectiveness of thyroxine in depressing the P/O ratio of oxidative phosphorylation in isolated liver mitochondria depends on the concentration of magnesium ion in the test medium(3). According to Tapley and Cooper(3), it is conceivable that interaction between thyroxine and magnesium may occur at critical sites within the mitochondrial structure.

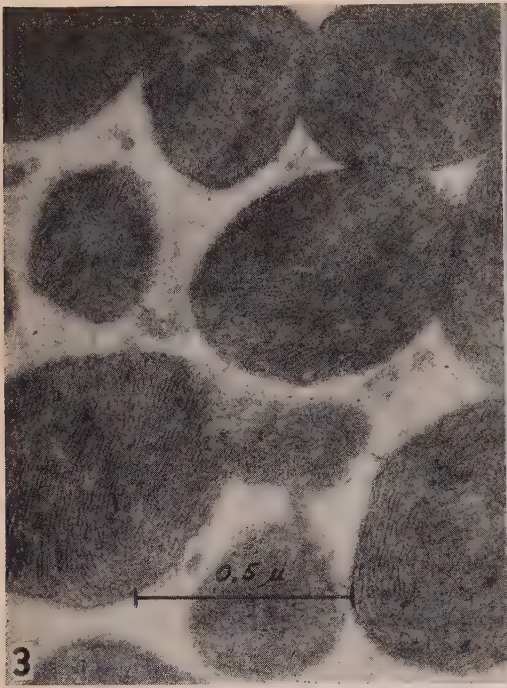
Dietary magnesium deficiency in rats produces a more marked decrease in P/O ratio of heart mitochondria than of liver and kidney mitochondria(1). The present study indicates that *in vitro* patterns of swelling of mitochondria from rats fed diets deficient in magnesium are similar to the spontaneous swelling in control rats when ATP and EDTA are added, but appear to be different from swelling induced by deficiency of essential fatty acids, in which case ATP does not prevent the swelling(11). The mitochondria of heart and liver from magnesium deficient rats respond very little, if at all, in the presence of thyroxine. *In vitro* swelling of mitochondria isolated from rats fed magnesium defi-

TABLE II. Phosphorus and Magnesium Content of Rat Heart and Liver Mitochondria Expressed in μmoles/g of Mitochondrial Nitrogen.*

Tissue	Group	Magnesium	Inorganic phosphorus
Heart mitochondria	Control rats	467 ± 43†	136 ± 11†
	Mg-def. rats	481 ± 96	144 ± 18
Liver mitochondria	Control rats	303 ± 34	332 ± 62
	Mg-def. rats	313 ± 26	331 ± 55

* Each value is mean and S.D. of 5 experiments of 2 rats each.

† Mean ± S.D.



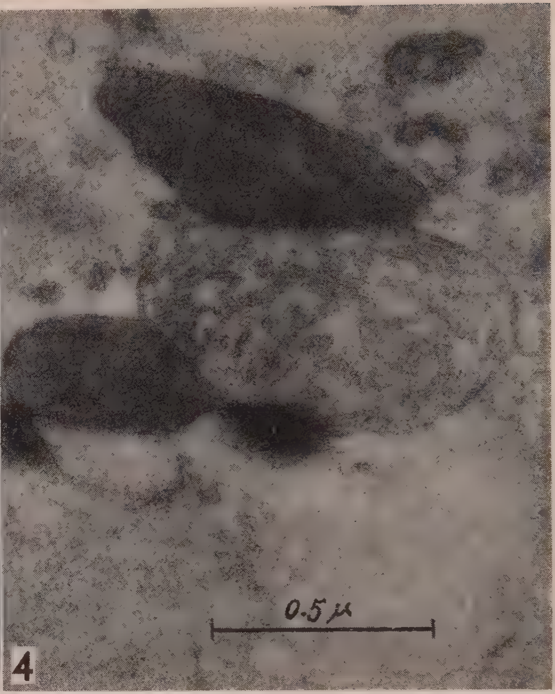


FIG. 3. Mitochondria of myocardium from control rat.

FIG. 4. Mitochondria of myocardium from rats fed a magnesium deficient diet, showing a swelling and disorganization of Cristae mitochondriales.

cient diets has *in vivo* significance, since electron microscopic examination of myocardial sections of magnesium deficient rats shows the mitochondria to be swollen with disorganization of the cristae mitochondriales.

It is unlikely that the higher rate of mitochondrial swelling in magnesium deficiency is due to a decrease in magnesium and/or to an increase in inorganic phosphorus in mitochondria, because magnesium and phosphorus levels were not changed in a statistically significant way. For these reasons, it was assumed that the primary effect of magnesium deficiency in causing a decrease P/O ratio may be an indirect effect mediated by some morphological membrane disorganization. Whether the increased swelling rate of mitochondria isolated from magnesium deficient rats is related to the probable loss of functional DPN as in the case of thyroxine induced swelling(5), or to some other cause such as changes in the calcium or fatty acids content(11), is being investigated further. As a preliminary result obtained in our laboratory, homogenate and mitochondria from the livers of rats fed a magnesium deficient diet for 10 days did not show any deficiency in respiratory control by phosphate acceptor. The relationship between the sensitivity of heart mitochondria to magnesium deficiency, the facts that swelling of heart mitochondria was not prevented by EDTA and that heart mitochondria contain almost twice the amount of magnesium found in liver mitochondria, remain as unsolved problems.

Summary. Swelling of mitochondria isolated from the heart and liver of rats fed a magnesium deficient diet was examined photometrically and by electron microscopy. A rapid swelling of heart and liver mitochondria was produced in rats fed a magnesium deficient diet for 10 days, whereas there was no significant decrease in the magnesium content of the mitochondria. A morphological swelling of heart mitochondria of rats fed a magnesium deficient diet was also demonstrated. Addition of thyroxine produced a slight increment or none in the rate of swelling of mitochondria isolated from deficient rats. ATP reversed swelling of heart and

liver mitochondria from deficient rats; EDTA, however, prevented only the swelling of liver mitochondria. Mitochondria of heart contained twice the amount of magnesium found in liver mitochondria. The mechanism of decrease of P/O ratio of mitochondria from magnesium deficient rats and the physiological significance of magnesium deficiency induced swelling of mitochondria, were discussed.

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Effects of LSD-25 on Food Intake in the Rat.* (26925)

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In an earlier study(1) of certain similarities between the effects of LSD-25 and amphetamine sulfate, a depression of body weight was observed in rats injected daily with 0.50 mg/kg of LSD-25. It seemed logical to suppose that this was the result of depression of food intake since Winter and Flataker(2) reported that at the height of the drug effect their rats did not eat. The present work quantifies this effect of LSD-25 and investigates the possibility of development of tolerance to daily doses of the drug.

Method and results. Experiment I. Thirty-six male albino rats of the Sprague-Dawley strain were trained for 18 days to feed 2 hours a day (2-4 p. m.) on powdered Purina Lab Chow. At the end of this period their mean body weight of 246 ± 3 g was approximately 80% of normal and they were assigned to one of 4 groups on the basis of mean amount of food intake over the last 3 training days. (Mean weights of the 4 groups did not differ significantly). Just before 2 p.m. on the 19th day, one of each of 3 groups was injected IP with one of the following doses of LSD-25: 0.13, 0.26, or 0.52 mg/kg. (LSD-25 was dissolved in Ringer's solution and doses prepared so that all animals received the same amount of fluid in proportion to body weight). The fourth group was injected with a comparable amount of Ringer's solution alone. Immediately after injection the animals were returned to their living cage, given cups of chow, and permitted to feed 2 hours. Four days of no injections and 2-hour feeding followed. On the fifth day each animal was again injected with the same dose level and permitted to feed 1½ hours. After another no-injection, 4-day interval with 2-hour feeding, the animals were again injected with the same doses and permitted to feed for one hour.

The data of this study (Fig. 1), show a depression of food intake as a function of LSD-25 dosage. A repeated measures analysis of variance(3) was run using the data of all 4 groups over the 3 injection days. This analysis yielded the following F ratios: be-

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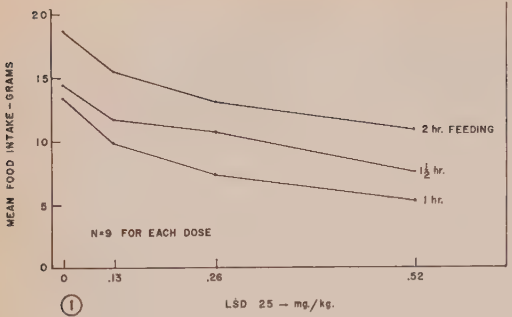
* The authors gratefully acknowledge the assistance of Wallace Clay in collecting data.



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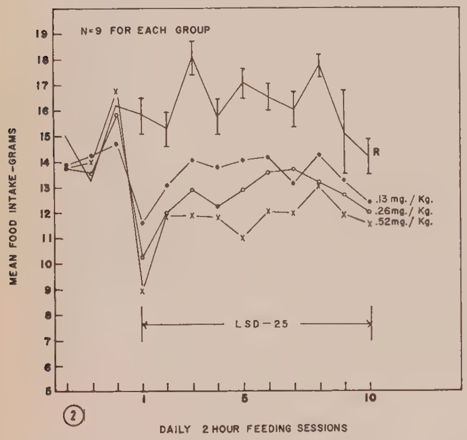

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FIG. 1. Food intake as a function of LSD-25 and time allowed to feed.

FIG. 2. Mean food intake (2-hr feeding) with daily inj. of LSD-25. Stand. error of means has been plotted for the Ringer's group.

tween methods = 68.5; between trials = 69.9; and interaction, trials and methods = 0.70. The first 2 were significant beyond the .01 level while the latter was not significant. These data reveal significant differences between dosage levels and time allowed to feed, but no differences between the shape of the curves generated by these 2 variables. The equation of the log dose relationship determined by analysis of the data for the first injection day (2-hour feeding) was, $Y = 16.2 + 7.5 \log X$.

Experiment II. In this study 36 male rats of the same strain were trained on the 2-hour, powdered chow feeding schedule for 14 days prior to first injection. On the first injection day these animals had a mean weght of 245 ± 4 g and were approximately 80% of normal body weght. Four groups were formed on the basis of mean food intake over the last 3 days of the training period (Mean weights of the 4 groups did not differ significantly). The same dosage levels of LSD-25 as used in Exp. I were investigated. Procedures were identical except that in this work all animals were injected and tested daily for 2 hours of feeding at 24-hour intervals for 10 days. These tests were run to determine whether or not the experimental groups would show a resumption of food intake at the level of the control group.

The results of this study (Fig. 2) show a drop in food intake with injection of LSD-25. A repeated measures analysis of variance of the 4 groups over the 10 injection days yielded the following F ratios: between methods = 7.43; between trials = 9.26; and interaction, trials and methods = 2.40. All of these ratios were significant beyond the .01 level. The between trials F appears to be related to the large increase in food intake in the experimental groups on the second injection day. Even so, none of the drug groups reached the control level at any period during the 10 days.

Discussion. These results show a log dose relationship between LSD-25 and depression of food intake but tolerance effects are not so obvious. A complete tolerance, in the sense that the experimental groups were back at the control level after 10 days, was obviously not obtained. Tolerance in the classical sense and defined as, "a phenomenon characterized by the fact that more and more of a drug must be used to produce equivalent effects"(4), was investigated only indirectly. For example, if tolerance developed over the 10 days of drug injection food intake of the experimental groups should have increased gradually, since dosage levels remained constant. In fact, the design of the study was such that it would tend to enhance any tolerance effects. As the groups given the drug were eating less than the controls they were becoming more deprived; this could have interacted with the drug effect to produce greater food intake in the drug groups. On the second day such an increase occurred, but the lack of any further increase contradicts such an interpretation. In fact, it was not until 5 days after the last injection (with the animals still on the 2-hour schedule but without injections) that the drug groups were eating as much as the controls. Whether this phenomenon was a direct, prolonged, drug effect, or the result of conditioned suppression of food intake, is not known. Conditioning could have occurred through pairing of the injection with presentation of food. This is an interesting hypothesis and one worth further investigation.

In previous work(1) investigating similarities between LSD-25 and amphetamine, rats injected with these substances showed increased speed in running to escape shock and increased incidence of avoidance of shock. The present work demonstrates still another similarity between the 2 drugs, although amphetamine was not investigated here since its anorexic effects are well known.

These present studies indicate that for assay of LSD-25, food intake is a reliable and sufficient dependent variable. They also serve as a warning on the use of food reinforcement in psychological testing of drug effects.

Summary. Suppression of food intake in the rat was shown to follow injection of LSD-25 and this effect was determined to be dose dependent. When the effects of 10 daily injections of the drug were investigated, a suppression of food intake on each day indicated a lack of development of complete tolerance. These data show that caution is needed in interpretation of the behavioral effects of LSD-25 where such behavior is dependent upon food reinforcement.

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The Zinc Content of Rat Sperm Cells from Ejaculate, Vas, Epididymis and Testis.* (26926)

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No biological role is known for the high concentrations of zinc in many tissues of the male mammalian reproductive tract, including human prostate(1), ejaculate(2) and sperm cells(3), canine prostate(4) and prostatic fluid(5), rat dorsolateral prostate and rabbit prostate(6). By mating male rats after ligation of the dorsolateral prostate, Gunn and Gould(7) showed that the zinc normally released by this gland is not needed for rat fertility or fecundity. If prostatic fluid were the sole source of zinc in the rat ejaculate, their data would virtually rule out a role for (male) zinc in rat fertilization. However, Wetterdal's data(8) suggest that there is another source of zinc. After injecting zinc-65 into rats he found high activity in the testes and an activity peak which traveled through the sperm transport system along with the spermatozoa that had been maturing in the testes at time of injection. A high concentration of zinc in rat sperm cells, independent of the prostatic fluid, is confirmed in the present paper by direct measurements on spermatozoa taken from ejaculates, vas, caudal epididymis and testis.

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[†] Present address: The Eldridge Reeves Johnson Foundation for Medical Physics, Philadelphia, Pa.

Methods. Ejaculated sperm cells were obtained from Wistar albino rats with electrical stimuli of 0-2 volts at an alternating current of 30 cycles per second, applied through a rectal probe(9). After ejaculation each rat was sacrificed and sperm cells were collected from the vas, caudal epididymis and testis of one side of the animal. Ejaculated copulatory plugs were obtained with peak voltages exceeding 2 volts; at lower peak voltages plugs do not form. The other tissues mentioned below were removed after sacrifice without prior ejaculation.

Sperm cells collected into Ringer's solution from ejaculates, vas and epididymis were washed in Ringer's and in water and then centrifuged at 2200 rpm (1000 g) for one hour. The sediments were placed on thin nylon films and vacuum-dried prior to zinc assay. Microscopic observations did not reveal any damage to the sperm cells (other than loss of motility) at any stage of the procedure preceding desiccation.

To obtain testicular sperm cells the testicular contents were first squeezed into Ringer's solution. After one hour at room temperature, most of the unwanted cells including immature sperm cells were settled at the bottom of the solution while the mature sperm cells, having become motile, were distributed throughout. The decanted solution was then centrifuged, yielding a residue which was predominantly sperm cells with some cellular contamination. This residue was washed in water, centrifuged again and prepared for zinc assay as described above.

Vas, dorsolateral and ventral prostates and other soft tissues were homogenized after excision in glass tube-and-pestle type tissue grinders. Homogenate samples were then mounted and dried as above.

As recommended by Sandell(10), water and Ringer's solutions were prewashed in a dilute solution of diphenylthiocarbazone ("dithizone") in carbon tetrachloride and rinsed in carbon tetrachloride to remove zinc contamination. Glassware was freed of zinc contamination by the same procedure, with an additional rinse in zinc-free water.

Zinc concentrations in the dried specimens were measured by a non-dispersive X-ray fluorescence instrument developed in this laboratory(11). Nearly monochromatic X-rays impinge on each specimen. A detector records simultaneously the scattered X-rays as an index of mass and the zinc X-rays as an index of zinc content. Zinc concentration (expressed in this paper as milligrams of zinc per gram of dried specimen) is obtained directly from these and calibration data. Sensitivity is high enough for convenient assays of sub-milligram specimens containing as little as 10^{-9} grams of zinc. Assays were generally run long enough to leave a probable statistical error of about 5%.

The adequacy of the zinc assay technic is shown in Table I, which summarizes a recovery experiment in which aliquots of a rat liver homogenate (7.5 g of rat liver in 37.5 cc of water) were supplemented with known amounts of zinc and copper sulfate dissolved in water. Copper was added because it is the one element which might be expected to interfere; a correction for this element, when necessary, is based on data obtained simultaneously from a second, suitably filtered detector. It is apparent that the method is accurate throughout the concentration range

TABLE I. Recovery Experiment.

Tube #	Added, cc	Added, dry mg	Added Zn, μg	Added Cu, μg	Nominal,* mg Cu/dry g	Nominal,* mg Zn/dry g	Measured, mg Zn/dry g
1	.99	.00	.0	.0			.097
2	.99	.06	25.0	.0	.038	.330	.328
3	.99	.25	100.	.0	.038	1.030	1.035
4	.99	.49	199.	.0	.038	1.950	1.980
5	1.98	.12	25.0	25.0	.280	.330	.334
6	1.98	.31	25.0	100.	.980	.330	.316

* Calculation of "nominal" concentrations is based on the data in the table and on measurements indicating that each aliquot, before supplementing, contained 107 dry mg, 10.4 μg of Zn and 4.1 μg of Cu in a volume of 2.50 cc.

TABLE II. Milligrams of Zinc per g of Dried Sperm Cells from Various Rat Tissues.

	Source of sperm cells			
Rat #	Testis	Caudal epididymis	Vas	Ejaculate
1	.33	.99	1.22	.79
2	.46	1.36	1.19	.96
3	.59	1.29	1.43	(lost)
4	.25	1.23	1.13	.38
5	.22	1.11	1.48	.85
6	.46	1.11	3.32	1.48
Avg	.39	1.18	1.29*	.89

* Rat #6 is omitted from the vas avg.

encountered in this report, and that the method of correction for copper is successful.

Results. Tables II and III list zinc concentrations in rat sperm cells taken from various sites, and in a variety of rat tissues.

Comparison of Tables II and III shows that an extraordinary concentration of zinc occurs in rat sperm cells independently of any contribution from prostatic or other fluid in the ejaculate.

Concerning Table II certain technical points should be noted. The most reliable values are for the vas and epididymal sperm cells. Microscopic inspection of the centrifuged material from these sources showed virtually no contamination. On the other hand, as might be expected, debris accompanied the centrifuged sperm cells from ejaculates and testes. This contamination may explain fully the lower average zinc level and higher variation in the ejaculate specimens. The contamination of the testicular material is at least as great, but there is also the possibility that the testicular sperm cells have not yet reached their ultimate zinc levels.

We do not know the reason for the odd high value for the sperm cells from the vas of animal #6; presumably the sample was inadvertently contaminated.

In order to see if the zinc levels remained unchanged through the periods of sedimentation and washing by centrifugation, spermatozoa were left at room temperature in water for extra periods up to one hour and in Ringer's solution for extra periods up to 3 hours before processing. Aliquots taken at different times during these periods showed no change in zinc levels. Repeated washings in water also had no effect.

In another "control" test to ascertain zinc retention during processing we compared the zinc level of the entire material squeezed from the vas with the level of centrifuged and washed sperm from the vas. The zinc level of the total material, comprising mainly sperm cells and vas fluid, was about 60% of the level of the processed sperm cells. The difference is due presumably to non-sperm material in the "total" specimen. The result certainly does not suggest any loss of zinc in processing of the spermatozoa.

Zinc was assayed also in specimens produced under 2 sets of electroejaculation conditions. In the copulatory plug produced with a peak stimulus above 2 volts concentration averaged 0.02 mg zinc/dried g. In the much smaller fluid specimen produced at lower voltage, after centrifugal removal of the sperm cells, concentration was 0.5 mg zinc/dried g.

For comparison, assays were run also on one canine electroejaculate. An aliquot of the whole ejaculate had 1.6 mg zinc/dried g and an aliquot after sperm cell removal (leaving only prostatic fluid) had 1.7 mg zinc/dried g. These 2 values are not significantly different. The average value of canine prostatic fluids is slightly less than 1.7 for a series run in our laboratory and to be published elsewhere, but the numbers 1.6 and 1.7 are listed in Table IV where the significance lies in their close similarity.

Discussion. Zinc is essential to many metal-

TABLE III. Milligrams of Zinc per g of Dried Rat Tissue.

Tissue	Present work*	Reference 3
Brain		.07
Epididymis	.22	.22
Heart	.10	.08
Kidney	.13	.11
Liver	.12	.11
Lung		.09
Muscle		.05
Pancreas	.07	.10
Dorsolateral prostate	1.00	.89
Ventral ”	.04	
Seminal vesicle	.09	
Spleen	.18	.09
Testis	.22	.21
Vas	.16	

* Prostate and vas: Avg of 6 animals. Other tissues: Only one animal.

TABLE IV. Zinc Concentrations (mg/Dried g) in Rat, Canine and Human Prostatic Tissues, Prostatic Fluids and Ejaculates.

	Material		
Species	Prostatic tissue	Prostatic fluid	Ejaculate
Rat	.60*[6]	.5? †	.02‡
Dog	1.2 [4]	1.6	1.7
Man	.9 [1]	7.2[5]	3.0[2]

* Dorsolateral prostate; the value for ventral is much less.

† Electroejaculate produced at low voltage, after sperm cell removal. Volume and zinc level suggest that this material is prostatic fluid.

‡ Electroejaculate produced with a peak stimulus above 2 volts.

loenzymes(12). Its concentrations in most soft tissues are in the neighborhood of 0.1 mg/dried g(13). The much higher zinc levels in certain parts of the mammalian reproductive tract suggest a special role for the element in fertilization.

As evidence against such a role Gunn and Gould(7) recently proved, by ligation of the dorsolateral prostate, that the presumably zinc-rich prostatic fluid is superfluous for rat fertility. As they noted however, any special need for zinc in the ejaculate may be met from a different source in the rat. Since an independent source of concentrated zinc is now established by our observations (Table II) on rat testicular, vas and epididymal sperm, the possibility of a role of high zinc concentrations in fertilization is still open.

If zinc is important in ejaculates (perhaps in sperm cells), the prostatic zinc contribution may be more important in dogs and in human beings than it is in rats. Table IV shows that canine and human prostatic fluids are more effective than rat prostatic fluid in raising the zinc level of the whole ejaculate. Canine prostatic fluid is not diluted in the ejaculate by contributions from other accessory sex glands, and human prostatic fluid supplies a truly extraordinary zinc concentration; on the other hand rat prostatic fluid is such a small fraction of the total rat ejaculate (14) that the zinc level in the latter is quite low. With respect to zinc metabolism, Table IV supports Gunn and Gould's view that the rat dorsolateral prostate is archaic and indicates that the prostate gland may be more important in dogs and human beings.

Summary. Zinc concentrations are unusually high in rat spermatozoa taken from testis, caudal epididymis and vas, as well as from the ejaculate. This finding supports the view that zinc may play a role in rat fertilization, despite the fact reported by others that the zinc-rich prostatic fluid is not necessary for fertility in that species.

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Influence of D-Thyroxine on Vascular Connective Tissue in Rabbits.* (26927)

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It has been demonstrated that l-thyroxine can induce alterations in the mucopolysaccharides of the aortic wall in rabbits(1) and intensify epinephrine-induced alterations(2). The aim of the present experiments was to investigate whether dextro-thyroxine, the optical isomer of levo-thyroxine, has a similar effect on the aortic wall. By injecting doses of d-thyroxine with an inhibitory effect on the release of thyrotrophic hormone equal to the l-thyroxine doses previously used(1), it was desired to demonstrate whether the thyroid hormone exerts its influence on the aortic wall by a direct peripheral action or *via* thyrotrophin inhibition.

Material and methods. Two separate experiments were carried out (with an interval of 10 months). Male albino rabbits weighing about 2 kg were kept on a uniform, adequate laboratory diet under regular control of body weight. In the *first experiment* one group of 22 rabbits received intravenous injections of epinephrine† daily for 11 days in doses increasing from .025 mg/kg to .050 mg/kg during the last 7 days. Simultaneously the animals were injected with 2 ml physiological saline intraperitoneally once a day. Another group of 31 rabbits received epinephrine as the previous group, and d-thyroxine,‡ suspended in physiological saline, .25 mg/kg once a day for 11 days injected intraperitoneally. In both groups one-fourth of the animals died before the 6th day and were excluded. The *second experiment* comprised 3 groups of rabbits: Twelve animals were injected with d-thyroxine .25 mg/kg intraperitoneally and physiological saline 1 ml intravenously daily for 2 weeks (corresponding to the epinephrine-injections in the first experiment). Eleven animals were injected with physiological saline 1 ml intravenously and 1 ml intraperitoneally daily for 2 weeks, while a third group of 10 animals served as untreated controls. The following technic was common for both experiments. Forty-eight hours before being sacrificed the animals received 1.5 mc of ^{35}S sulfate§ intraperitoneally without regard to body weight. A correction for weight was made after the final measurements. The rabbits were sacrificed by air embolism, the entire aorta and the heart removed and the hearts weighed. The gross alterations in aortae were estimated by grading from 0-4 as in previous experiments (1,2). Specimens for microscopic examinations removed from the arch, the thoracic and the abdominal part of aorta were fixed in a 4% aqueous solution of lead subacetate. Sections were stained with hematoxylin-eosin (Harris), orcein (Unna), the van Gieson-Hansen connective tissue stain and with a .5% aqueous solution of toluidine blue for metachromasia. In the first experiment each aorta was divided into 3 parts for biochemical investigation: I from the aortic valve to the first intercostal arteries, II from the first intercostal arteries to the diaphragm and III the abdominal aorta. In the second experiment the whole aorta was analysed as one. The adventitia was stripped off. Water content was determined after drying in a vacuum desiccator for 48 hours at room temperature. Thereupon the tissue was homogenized as described previously(2). Total content of mucopolysaccharides was estimated by determination of the content of hexosamine by the method of Elson and Morgan(3) as modified by Boas(4) and later by Kirk and Dyrbye(5) and Dyrbye(6). The content of hydroxyproline, representing the amount of col-

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* Supported by grants from Danish State Research Foundation and Danish Foundation for Advancement of Medical Science.

† A .01% solution of epinephrine hydrochloride.

‡ Generously supplied by Travenol Laboratories Inc., Morton Grove, Ill., as d-thyroxine-sodium.

§ Carrier-free ^{35}S sulfate obtained from the Radiochemical Centre, Amersham, England. Diluted to a suitable volume with a 10 mg per 100 ml solution of sodium sulfate in physiological saline.

TABLE I. Content of Hexosamine and Hydroxyproline in Rabbit Aortae, μg/mg Dried Tissue and Hexosamine/Hydroxyproline Ratio Following Injection of Epinephrine plus d-thyroxine and Epinephrine plus Physiological Saline.

Group	I§	II	III	IV	P values‡ I-II	I-III	II-III
			Hexosamine				
Epinephrine + d-thyroxine	8.9 ± .35* (20)†	7.9 ± .23 (20)	8.2 ± .40 (17)	8.4 ± .26 (17)	<.05	n.s.	n.s.
Epinephrine + physiol. sal.	8.0 ± .45 (15)	6.9 ± .29 (14)	7.6 ± .29 (13)	7.4 ± .32 (13)	<.05	n.s.	n.s.
P values	n.s.	<.02	n.s.	<.05			
			Hydroxyproline				
Epinephrine + d-thyroxine	21.3 ± .53 (20)	20.9 ± .76 (23)	23.5 ± 1.6 (20)	21.9 ± .62 (18)	n.s.	n.s.	n.s.
Epinephrine + physiol. sal.	21.3 ± .66 (15)	21.6 ± .56 (16)	24.9 ± .87 (15)	21.8 ± .55 (15)	n.s.	<.01	<.01
P values	n.s.	n.s.	n.s.	n.s.			
			Hexosamine/hydroxyproline ratio				
Epinephrine + d-thyroxine	.42 ± .024 (20)	.37 ± .017 (20)	.36 ± .021 (16)	.38 ± .019 (16)	n.s.	<.05	n.s.
Epinephrine + physiol. sal.	.38 ± .026 (15)	.32 ± .019 (14)	.30 ± .016 (13)	.34 ± .017 (13)	n.s.	<.05	n.s.
P values	n.s.	n.s.	n.s.	n.s.			

* Mean ± stand. dev. of mean.
† No. of samples.
‡ Statistical significance was determined by comparing mean values in Students' t test.
§ I-III—different parts of aorta, IV—aorta as a whole, calculated on basis of total wt of I-III.

lagen, was determined by the method of Neuman and Logan(7) modified by Martin and Axelrod(8). The exchange of ester-bound sulfate in sulfated mucopolysaccharides was estimated by determination of the *in vivo* uptake of ^{35}S sulfate after ashing of the tissue with fumic nitric acid and precipitation of the sulfur as barium sulfate as devised by Moltke

TABLE II. Uptake of ^{35}S Sulfate in Counts/Min./10 mg Dried Tissue in Aortae and Skin, and Content of Water, % of Wet Weight in Aortae of Rabbits Injected with Epinephrine plus d-thyroxine and Epinephrine plus Physiological Saline.

	^{35}S sulfate uptake						
	Aorta				Skin		
Group	I‡	II	III	IV			
Epinephrine + d-thyroxine	79 ± 7* (16)†	69 ± 8 (16)	80 ± 11 (15)	74 ± 7 (15)	31 ± 4 (15)		
Epinephrine + physiol. sal.	56 ± 6 (13)	44 ± 3 (13)	61 ± 7 (13)	51 ± 5 (11)	39 ± 5 (13)		
P values	<.05	<.02	n.s.	<.05	n.s.		
	Content of water				P values		
					I-II	I-III	II-III
Epinephrine + d-thyroxine	75.4 ± .40 (23)	72.6 ± .65 (23)	72.2 ± .89 (22)	73.7 ± .46 (22)	<.001	<.01	n.s.
Epinephrine + physiol. sal.	74.9 ± .84 (16)	72.2 ± .85 (15)	70.3 ± 1.6 (16)	72.8 ± .84 (15)	<.05	<.02	n.s.
P values	n.s.						

* Mean ± stand. dev. of mean.
† No. of samples.
‡ I-III—different parts of aorta, IV—aorta as a whole.

(9). Activity was measured at infinite layer-thickness with a Geiger-Müller tube, counting to a statistical error of about 3%. The *in vivo* uptake of ^{35}S sulfate in the skin was also determined. The dose of d-thyroxine was 5 times as large as the dose of l-thyroxine used in the previous experiment(1) to obtain a similar suppression of the thyroid stimulating hormone (TSH) production. The inhibitory effect of d-thyroxine on endogenous production of TSH is estimated to about one-third of that of l-thyroxine(10). In a preliminary experiment the inhibitory effect of l- and d-thyroxine on release of thyroid hormone from the thyroid gland was compared in 4 rabbits injected intraperitoneally with .05 mg/kg l-thyroxine and 4 with .25 mg/kg d-thyroxine daily, using ^{131}I and daily counting over the thyroid gland with a scintillation counter, recording a "release curve" as described by Brown-Grant(11).

Results. In the *first experiment* the rabbits injected with epinephrine plus d-thyroxine averaged 8.0% loss in weight in contrast to a weight gain of 4.5% in the epinephrine-saline group. The weight of the hearts expressed in per cent of body weight was highest in the epinephrine-d-thyroxine group (.36 ± .009 and .30 ± .010 g respectively), whereas absolute weights did not differ. Two-thirds of the epinephrine-d-thyroxine injected rabbits showed rather pronounced gross alterations (grade 2-3) in aorta I and II in contrast to slighter alterations (grade 1-2) in half of the epinephrine-saline injected animals. The microscopic lesions consisted of calcified media lesions, stretching and splitting of the elastic membranes, accumulation of amorphous metachromatic substance between the elastic membranes and intimal thickening, most pronounced in the epinephrine-d-thyroxine group and similar to the alterations previously described(1,2,12). The content of hexosamine in part II of aorta (Table I) and the uptake of ^{35}S sulfate (Table II) in part I and II was highest in the epinephrine-d-thyroxine group. In the *second experiment* all 3 groups gained weight, but the d-thyroxine group less than the 2 others (Table III). No gross alterations were seen in the aortae except for a few small

TABLE III. Alterations in Body Weight and Weight of Hearts, Content of Hexosamine and Hydroxyproline, μg/mg Dried Tissue, Hexosamine/Hydroxyproline Ratio, Content of Water in % of Wet Weight in Rabbit Aortae and Uptake of ^{35}S Sulfate in Counts/100 Sec./10 mg Dried Tissue in Aortae and Skin Following Injections of d-thyroxine plus Physiological Saline, Physiological Saline and in Untreated Controls.

Group	Alterations in body wt, % of initial wt	Wt of hearts		He	Hp	He/Hp	Water content	^{35}S sulfate uptake	
		g	% of body wt					Aortae	Skin
1. d-thyroxine + physiol. sal.	6.8 ± .63* (12)†	4.54 ± .24 (12)	.22 ± .010 (12)	7.1 ± .14 (12)	21.4 ± .56 (12)	.34 ± .014 (12)	69.9 ± .26 (12)	274 ± 21 (12)	139 ± 15 (11)
2. Physiol. saline	12.7 ±2.0 (11)	5.19 ± .23 (11)	.21 ± .005 (11)	7.5 ± .18 (11)	21.0 ± .76 (11)	.36 ± .009 (11)	70.8 ± .30 (11)	203 ± 10 (11)	157 ± 22 (10)
3. Untreated controls	12.8 ±1.1 (10)	4.73 ± .20 (10)	.20 ± .005 (10)	7.1 ± .22 (10)	21.7 ± .52 (10)	.33 ± .014 (10)	71.0 ± .50 (10)	193 ± 15 (10)	143 ± 19 (9)
P values 1-2	<.01						<.05	<.01	
1-3	<.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<.01	n.s.
2-3	n.s.						n.s.	n.s.	

† No. of samples.
* Mean ± stand. dev. of mean.

white nodules just above the aortic valve in a few rabbits in all 3 groups. Except for these lesions, microscopically visible as subintimal, fibrous calcified lesions, none of the animals showed severe medial alterations, but half of the d-thyroxine-injected and 2 of the saline-injected animals had scattered accumulations of metachromatic substance in the media between the elastic membranes. Considering the content of water, hexosamine, hydroxyproline and the hexosamine/hydroxyproline ratio no difference was seen between the 3 groups, whereas the uptake of ^{35}S sulfate in aorta showed a significant increase in the d-thyroxine group. The injected doses of d- and l-thyroxine were found to have a complete inhibitory effect on release of ^{131}I-labelled hormone from the thyroid gland. The effect was noted 24 hours after onset of injections.

Discussion. D-thyroxine showed an influence on the aortic wall similar to that of l-thyroxine(1,2). It enhanced the damaging effect of epinephrine and induced alterations in the mucopolysaccharides of the arterial wall. The alterations observed might accordingly be interpreted as a repair process elicited by injury to the arterial wall(2). However, even if injected in 5-fold higher doses, d-thyroxine alone in contrast to l-thyroxine (1), was unable to induce quantitative alterations in the hexosamine content. As the doses of d- and l-thyroxine exerted an equally inhibitory effect on release of thyrotrophic hormone, it seems reasonable to assume, that d-thyroxine as well as l-thyroxine exerts its influence on the arterial wall by a direct peripheral action and not *via* TSH.

Summary. D-thyroxine enhanced epinephrine-induced alterations in the aortic wall in rabbits, causing an increase in hexosamine content and uptake of ^{35}S sulfate. D-thyroxine alone increased uptake of ^{35}S sulfate. Compared to l-thyroxine used in previous experiments, d-thyroxine acted similarly but to a lesser extent. As the applied amounts of d- and l-thyroxine exerted an equally inhibitory effect on TSH, d-thyroxine as well as l-thyroxine probably exerts its damaging effect on the arterial wall by direct peripheral action.

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Cytotoxicity of Adrenal Cortex Hormones on Normal and Malignant Lymphocytes of Man and Rat.* (26928)

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I have shown by the method of unstained cell counts(1) that cortisol and related hormones have an *in vitro* cytotoxic effect on cells derived from the rabbit thymus. The newly developed slide-chamber method(2) provided a means for extending this study to human lymphocytes. This method was used in this study to measure the toxic effect of cortisol on normal human and rat lymphocytes and also on human leukemic lymphocytes.

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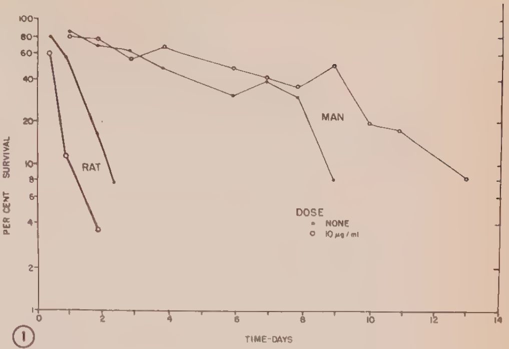
Methods. Three groups of individuals were used in these studies. First was a group of 7 normal men and women, employees of the Research and Laboratory services, to whom I am indebted. A second group consisted of 16 non-leukemic, ambulatory, male hospitalized patients with more or less normal hemograms. Specimens from these patients were obtained through courtesy of the Laboratory Service of Hines Hospital. A third group consisted of 12 men and 1 woman with chronic lymphocytic leukemia or lymphosarcoma in leukemic phase. Specimens from these patients were obtained from the Hematologic Sections of the Medical Service of Hines Hospital and of Hektoen Clinic of Cook County Hospital.

The leukocytes from human or rodent blood were concentrated, washed, and resuspended in equal parts of normal homologous serum and tissue culture medium 199 (Difco). Cell suspensions were also prepared from a normal human spleen and a lymph node. The cortisol succinate was the commercial preparation Solu-Cortef (Upjohn). The cell suspension plus hormone was put up in slide-chambers(*2*) composed of 2 large cover slips separated by a metal disk 0.9 mm in thickness. The slide-chambers were incubated at 37°C.

The numbers of viable lymphocytes were counted one or more times a day in an area 0.04 × 10 mm by means of an inverted phase microscope with special accessories. The criteria for viable lymphocytes have been described(*2*). The counts enabled the construction of survival curves for treated and untreated lymphocytes. Survival curves were summarized by the index, the 10% survival time; *i.e.,* time in days during which 90% of the lymphocytes died or were killed and 10% survived.

The effect of a reagent was calculated by the arbitrary formula: the logarithm of 10% survival time of untreated lymphocytes minus that of 10% survival time of treated lymphocytes. An index of 0 means no effect of the reagent at a specific dosage. The same index was used as a measure of sensitivity of the lymphocytes to the reagent at a given dose.

Results. Rat blood lymphocytes. Suspensions of rat blood lymphocytes were incubated with and without cortisol succinate (10 μg/ml). Fig. 1 presents survival curves of rat blood lymphocytes in a typical experiment. It is seen that the viable lymphocytes in the control suspension decreased slowly and that 10% of rat lymphocytes survived 2.14 days. In contrast, the 10% survival time was only 0.90 days for lymphocytes treated with 10 μg/ml.



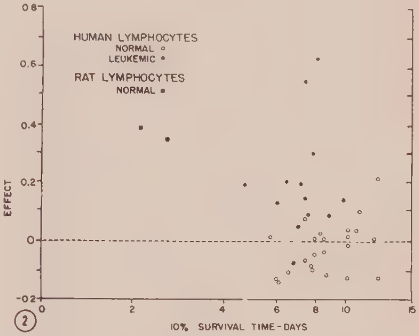


FIG. 1. Survival curves of rat and human lymphocytes incubated with and without cortisol succinate, 10 μg/ml. Abscissa represents days of incubation; ordinates, logarithms of percentage of lymphocytes surviving incubation.

FIG. 2. Effect of 10 μg/ml of cortisol succinate on lymphocytes from the blood of 23 non-leukemic individuals (○), of 13 leukemic patients (●), and of 2 rats (■). Abscissa represents 10% survival time of untreated suspension; ordinates, the effect as measured by the formula given under *Methods.*

The effect of varying doses of cortisol succinate on rat lymphocytes is shown in Table I. It is seen that 0.1 μg/ml produced a moderately toxic effect. Increasing the dosage to 100 μg/ml produced little, if any, increase in toxicity. A dosage of 1000 μg/ml was, however, highly toxic.

Human blood lymphocytes. The effect of

TABLE I. Effect of Adrenal Cortical Hormones on Normal Lymphocytes from Human and Rat Blood.

Blood lymphocytes from	Reagent	Effect* Dose (μg/ml) 1000	 100	 10	 1	 .1	 .01
Rat	Cortisol succinate	.75	.45	.38	.34	.39	.16
	Cortisol		.35	.23	.22	.24	.13
	Corticosterone		.58	.30	.26	.16	.05
Man	Cortisol succinate	.55	.03	−.06	−.09		
	Cortisol		.20	.14	.02		
	Corticosterone		.14	.04	.01		

* Effect = log (10% survival time of untreated lymphocytes) minus log (10% survival time of treated lymphocytes).

cortisol succinate on blood lymphocytes of a patient is shown in Fig. 1. The patient had a normal hemogram. The control untreated lymphocytes in 50% human serum had a 10% survival time of 8.7 days, much longer than that observed for rat blood lymphocytes in 50% rat serum. The long 10% survival time of human lymphocytes as compared to that of rat lymphocytes has been observed previously(3). The lymphocytes treated with 10 μg/ml of hormone had a 10% survival time of 12.3 days, 34% greater than the control. Cortisol succinate caused, if anything, longer survival of the cells. According to these experiments, 10 μg/ml had a toxic effect on rat lymphocytes but not on human lymphocytes. The sensitivity of rat and human lymphocytes is also compared in Table I. A dose as small as 0.01 μg/ml was toxic to rat blood lymphocytes but doses of 10 and 100 μg/ml were not appreciably toxic to human cells.

Fig. 2 and Table II summarize the effect of 10 μg/ml on blood lymphocytes of 16 non-leukemic patients and 7 normal individuals. The average effects were −0.04 and −0.02 respectively, which are not significant (Table II). In only one test, a positive effect of 0.20 was observed (Fig. 2). However, a repeat test on this individual failed to substantiate significant sensitivity of the lymphocytes to the hormone.

It may be concluded that a dosage of 10 μg/ml of cortisol succinate had no perceptible cytotoxic effect on human blood lymphocytes of non-leukemic individuals. In contrast, rat blood lymphocytes were sensitive to dosages as low as 0.01 μg/ml.

Human leukemic lymphocytes. Thirteen patients with chronic lymphocytic leukemia or with leukemic lymphosarcoma were tested one or more times for the sensitivity of their blood lymphocytes to cortisol succinate (Table II and Fig. 2). The table shows that 10 μg/ml of cortisol succinate was slightly but significantly toxic to the lymphocytes of 13 leukemic patients but was not toxic to lymphocytes of 23 non-leukemic individuals. Fig. 2 shows that the results varied from no sensitivity for leukemic lymphocytes of 4 patients to moderate sensitivity (0.53 and 0.60) for the cells of 2 patients. Six repeat tests were done on 5 leukemic patients. The effect found on a second and third test deviated from the original test by 0.010 to 0.101. Average deviation was 0.044. In other words, the leukemic lymphocytes remained as sensitive to cortisol succinate on a repeat test as on the first test.

Other tissues and other corticosteroids. A normal human spleen and a lymph node were available for testing. The spleen was removed incidentally during a partial gastrectomy for a gastric ulcer. A suspension prepared from the spleen consisted largely of normal lymphocytes. Lymphocytes of the spleen and lymph node were as resistant to cortisol succinate as blood lymphocytes (Table II).

The toxic effects of other corticosteroids on rat and human blood lymphocytes were studied. The free steroids, cortisol and corticosterone, were dissolved in methyl alcohol. 0.1 cc of the required dilution of these steroids was placed in the center of a large cover slip. The alcohol was allowed to evaporate

TABLE II. Effect of 10 μg/ml of Cortisol Succinate on Human and Rat Lymphocytes in 50% Homologous Serum.

Source of lymphocytes	No. of individuals or animals	Avg 10% survival time (days)		Effect* (avg)	Stand. error
		Untreated lymphocytes	Lymphocytes + reagent		
Rat blood	6	2.43	1.43	.282	.045
Human blood					
Normal individuals	7	9.31	9.86	−.042	.049
Non-leukemic patients	16	8.78	9.36	−.024	.017
Leukemic patients†	13	7.73	5.14	.194	.053
Human					
Lymph node—normal	1	5.75	5.56	.015	
Spleen— ”	1	8.78	8.90	−.006	

* See footnote, Table I.

† Diagnoses: Chronic lymphocytic leukemia and lymphosarcoma in leukemic phase.

leaving a fine deposit. The cell suspension was then added to the precipitated steroid on the cover slip and the slide-chamber was completed and sealed. Both cortisol and corticosterone were toxic to rat lymphocytes (Table I). On the other hand, survival of human lymphocytes was not appreciably affected by moderate doses of cortisol or by corticosterone.

Discussion. Dougherty and White(4) demonstrated that injections of adrenal cortical hormones and adrenocorticotropin into mice and rats resulted in lymphopenia and atrophy of lymphoid tissue. The findings have been amply confirmed by others, as indicated by Valentine *et al.*(5). The *in vivo* findings stimulated many *in vitro* studies. Corticosteroids were found to have an *in vitro* cytotoxic effect on rat and rabbit lymphocytes according to the unstained cell count method(1), tissue culture(6) and, in the present study, the slide-chamber method. The *in vitro* and *in vivo* findings agree that rat lymphocytes are sensitive to glucocorticoids.

Results of studies by different workers on *in vivo* effects of glucocorticoids on lymphocytes in man have been variable(5,7,8). According to a review by Valentine *et al.*(5), it has not been conclusively demonstrated that adrenal cortical hormones produce lymphopenia in man. The authors, therefore, raised the question whether hematologic experiments with corticosteroids on mice and rats can be extrapolated to man. Similarly Long (9) concluded that there are cortisone-sensitive and cortisone-resistant species. Long's conclusion was based on reports of *in vivo* effects of cortisone on body weight, gamma globulin synthesis, and antibody production. According to these *in vivo* criteria, the rat and rabbit are cortisone-sensitive and man is cortisone-resistant.

The present *in vitro* work showed that glucocorticoids in small doses were cytotoxic to blood lymphocytes of the rat but had no perceptible effect on blood lymphocytes of 23 men. Both *in vitro* and *in vivo* work indicates that rat lymphocytes are sensitive to glucocorticoids but human lymphocytes are resistant.

It seems of particular interest that cortisol succinate was, on the average, more cytotoxic to leukemic than to normal lymphocytes. In contrast, several other reagents, including x-rays and nitrogen mustard were, on the average, equally toxic to normal and leukemic lymphocytes(10). However, the sensitivity of the leukemic lymphocytes to the steroid was usually only slight. Only 2 of 13 patients had leukemic lymphocytes that were moderately sensitive.

Shaw *et al.*(11) administered prednisone to 18 patients with chronic lymphocytic leukemia. The therapy usually resulted in an increase in lymphocyte count of the blood, suggesting that the lymphocytes of the blood of the leukemic patients were not sensitive to prednisone therapy.

Shaw *et al.*(11) also found that 2 of 18 patients showed a decrease in blood lymphocytes as a result of prednisone therapy. In the present study, 2 of 13 patients had lymphocytes which were moderately sensitive to cortisol succinate. The parallel *in vivo* and *in vitro* findings suggest further studies to determine whether there is a correlation between *in vitro* sensitivity of leukemic lymphocytes to cortisol and *in vivo* response of the leukemic patient.

Summary. Cortisol succinate, cortisol, and corticosterone in small doses were toxic to lymphocytes from rat blood and rabbit thymus. The toxicity was indicated by a reduction in *in vitro* survival time of the lymphocytes, according to a slide-chamber method. Minimal toxic effects were produced by 0.01 μg/ml of cortisol succinate and cortisol and by 0.1 μg/ml of corticosterone. In contrast, lymphocytes from the blood of normal individuals and from non-leukemic patients were resistant to 10 μg/ml of cortisol succinate. These experiments suggest a species difference in the sensitivity of lymphocytes from man as compared to those from the rat and rabbit.

Similar tests were done on the lymphocytes from the blood of 13 patients with chronic lymphocytic leukemia or with lymphosarcoma in leukemic phase. In general, the leukemic lymphocytes were more sensitive to cortisol succinate (10 μg/ml) than normal lymphocytes. Two of the 13 patients had lymphocytes which were very sensitive to the corticosteroid.

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Experimental Phenylketonuria in Rats.* (26929)

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Auerbach, Waisman and Wyckoff(1) found that rats fed a diet containing 5% DL-phenylalanine or 2.5% each of DL-phenylalanine and L-tyrosine showed a 2-fold increase in plasma phenylalanine levels and an increased excretion of urinary phenylpyruvic acid. Similar observations were reported recently by Huang *et al.*(2). However, Neill and Langford(3) failed to demonstrate any phenylketonuria in rats fed a synthetic diet containing as high as 3.75% each of L-phenylalanine and L-tyrosine. The slightly elevated plasma phenylalanine levels obtained by previous investigators(1,2) were much lower than those found in phenylketonuric patients or in experimental phenylketonuric monkeys(4,5). The increased excretion of phenylpyruvic acid by rats fed DL-phenylalanine(1,2) is in all probability due to presence of the D-isomer, since D-phenylalanine can be deaminated by D-amino acid oxidase in kidney to form phenylpyruvic acid(6). Therefore, it seemed appropriate to obtain additional information in rats fed excessive quantities of the L-isomer of phenylalanine before the observations made in rats, monkeys and man could be compared biochemically.

Methods and materials. Eighteen-day-old

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TABLE I. Individual[*] Daily Food Intakes of Rats Kept in Normal and in Light-Dark Rooms.

	Normal		Light-Dark	
	Total 24 hr	8:00 a.m.-3:00 p.m.	Total 24 hr	8:00 a.m.-3:00 p.m.[†]
	g			
	16.4	.0	14.3	1.4
	16.1	.9	15.3	3.9
	19.4	1.4	17.5	4.9
	20.0	.4	16.9	3.6
	16.2	.2	17.2	3.0
	18.4	.0	18.4	3.3
	19.4	1.4	13.8	1.7
	17.0	1.3	18.7	4.1
	18.6	.6	20.2	4.9
	17.2	.8	17.5	6.5
Avg	17.9	.7	17.0	3.7

[*] These values are avg of 3 consecutive days' intake of individual rat.

[†] The time-switch in Light-Dark room was so arranged that the 3 hr dark period occurred from 11:00 a.m. to 2:00 p.m.

male rats[†] were fed a ground commercial chow diet with or without L-phenylalanine. The animals were kept in individual cages either in an ordinarily window-lighted animal room or in a dark room equipped with an automatic timeswitch set to have alternate 3 hour light and dark periods throughout 24 hours. This is referred to as the "light-dark room".

Plasma phenylalanine was determined by the method of Udenfriend and Cooper(7). Urine samples were tested qualitatively for phenylketones with 10% ferric chloride or with saturated 2,4-dinitrophenylhydrazine or by the Phenistix[‡] method. Quantitative analyses for phenylpyruvic acid were made by the method of Berry and Woolf(8). The phenylalanine hydroxylase activities of the rat livers were determined by the method of Udenfriend and Cooper(9). Food intakes were recorded several times daily at the desired intervals.

Results and discussions. It was previously noted in experimental phenylketonuria in monkeys that total daily intake of phenylalanine, as well as frequent feedings of this amino acid were 2 important factors necessary to produce sustained high plasma phenylalanine levels which in turn caused excretion of urinary phenylpyruvic acid. Since it is known that rats are nocturnal feeders the light-dark room was used in order to increase the frequency of feedings in the rats.

Table I presents daily food intakes of 2 groups of 10 rats fed a normal diet and kept either in a normally-lighted room or in the light-dark room. No significant difference in total daily food intake was observed between these 2 groups. However, the rats kept in the normal room consumed only about 4% of their total intake during the day (8:00 a. m. to 3:00 p. m.) whereas the animals kept in the light-dark room consumed 21% of their intake during the same period which included 3 hours of darkness. The results showed that rats consumed their food mainly during the dark, and that the light-dark cycle stimulated a more frequent intake.

Rats fed diets containing high phenylalanine appeared to change their eating pattern as shown in Table II. Total 24 hour intake remained the same in the 2 rooms at any percentage of phenylalanine added to the diet. The rats fed the higher levels of phenylalanine consumed less diet than at lower levels and these rats ate frequently despite darkness or light in contrast to rats fed normal diet or diets containing less phenylalanine. These changes in food intake cannot now be completely explained, but in all probability are a reflection of the high phenylalanine intake.

Table III presents ranges of plasma phenylalanine values of rats together with body weight and daily phenylalanine intake as af-

TABLE II. Food Intakes[*] of Rats Fed Diet Containing L-Phenylalanine Kept in Normal or in Light-Dark Rooms.

	Normal		Light-Dark	
Phenylalanine in diet, %	Total 24 hr	8:00 a.m.-3:00 p.m.	Total 24 hr	8:00 a.m.-3:00 p.m.
	g			
.0	17.9	.7 (3.9)[†]	17.0	3.7 (20.9)
3.5	14.0	1.2 (8.5)	12.8	2.6 (20.2)
5.0	6.2	1.1 (17.7)	6.6	1.5 (22.8)
7.0	8.1	1.9 (23.5)	8.7	2.3 (26.5)

[*] These values are avg intake of 10 animals for 3 consecutive days at end of 3rd wk except for the group fed 5% L-phenylalanine diet which was recorded at end of first wk.

[†] Values in parentheses expressed as % of total 24 hr intake.

[†] Holtzman Rat Co., Madison, Wisc.

[‡] Ames Co., Elkhart, Ind.

TABLE III. Correlation of Body Weight and Phenylalanine Intake with Plasma Phenylalanine Levels.

Phenylalanine in diet, %	Duration of exp., wk	Body wt, g*	Daily phenylalanine intake/100 g body wt, g†	Range of plasma phenylalanine, mg/100 ml		Activity§ of liver phenylalanine hydroxylase
				Midmorning	Midafternoon	
			Normal room			
0	1	62.0	.06	.9– 1.8‡	.9– 1.8	
	2	101.3	.06			
	3	151.7	.05			
3.5	2	113.0	.44	2.5– 2.7	1.0– 2.2	
5.0	1	52.0	.60	4.1– 9.9	3.5– 9.9	
	2	84.0	.57	2.2– 5.5	2.8–14.0	
7.0	1	36.0	.74	25.8–38.0	23.4–26.0	
	3	58.0	.98	12.0–34.0	16.5–29.0	
			Light-dark room			
0	1	68.0	.07	.9– 1.8	.9– 1.8	6.7 ± .3‖
	2	100.6	.06			7.3 ± .3
	3	152.3	.04			7.6 ± .3
3.5	2	100.6	.42	2.3– 5.3	3.1– 4.4	3.0 ± .5
5.0	1	50.0	.81	16.5–19.2	20.5–21.1	
	2	76.6	.60	8.2–21.2	6.2–10.7	
7.0	1	39.0	.98	18.2–70.6	17.5–30.2	3.3 ± .5
	3	52.9	1.15	16.0–36.0	12.0–28.8	4.5 ± .8

* Avg initial body weights are 38.5–40.5 g. Ten rats in each group.
† Values based on final body wt and avg daily intake of last 3 days of experiment.
‡ Range of all normal animals studied.
§ μM of tyrosine formed/hr/g of wet liver at 25°C.
‖ Stand. error of mean.

fected by diets containing 3.5, 5, or 7% of the amino acid. Blood samples of some rats were taken in the midmorning and others in the midafternoon to insure an accurate observation of changes in plasma phenylalanine levels as affected by diet. These data indicate that 3.5% L-phenylalanine diet neither affected growth of rats nor influenced plasma phenylalanine values when the animals were kept in the normal room, whereas a slight increase in plasma phenylalanine levels was noted in the animals kept in the light-dark room. It will be remembered that this difference may be due to the frequent feedings of the animals kept in light-dark rooms (Table II).

Rats fed the 5% L-phenylalanine diet did not grow as well as those on normal diet. Significantly higher plasma phenylalanine values than normal were observed in these animals regardless of the room. However the animals kept in the light-dark room appeared to have more consistently elevated plasma phenylalanine levels. Presumably when rats receive various quantities of phenylalanine orally, plasma phenylalanine levels of these animals reach a peak within a short time (less than half hour, unpublished data) then gradually return to normal values. Therefore, animals which receive repeated small dosages of phenylalanine (by frequent feedings in a light-dark room) may not have as high a peak value of plasma phenylalanine but will exhibit persistently elevated plasma phenylalanine values as compared with rats which receive one large feeding of the compound.

Prolonged and extraordinarily high plasma phenylalanine values comparable to those of phenylketonuric patients and monkeys were found in rats receiving the diet containing 7% L-phenylalanine despite the room in which the animals were housed. High plasma phenylalanine values were observed not only in samples taken in the morning but also in samples taken in the afternoon. From the eating patterns of the animals (Table II) it was no surprise that high plasma phenylalanine could be maintained throughout the whole day. However, growth of these rats was adversely affected by this highest level of L-phenylalanine and 10% mortality was noted in some groups during the first week.

The extraordinarily high plasma phenylalanine values of rats receiving 7% L-phenylalanine may be simply the result of overloading the metabolic capacity of the phenylalanine hydroxylase enzyme system. Freedland, Krakowski and Waisman(10) indicated that excess dietary phenylalanine decreased liver phenylalanine hydroxylase activity of rats kept in a normal animal room. The present results show that lowered phenylalanine hydroxylase activity by excess dietary phenylalanine was also obtained in the rats kept in a light-dark room (Table III). The activity of this enzyme was reduced to 40-60% of the value in the controls regardless of concentration of the amino acid and duration of experiment. The rats grew less on high phenylalanine diets; liver weight expressed as percentage of body weight remained the same. The phenylalanine hydroxylase activity therefore would always be lower in experimental animals compared to normal controls whether the activity was expressed per 100 g body weight or per total liver.

The results in Table III also indicate that concentration of dietary amino acid was more important to attain high plasma phenylalanine values than duration of experiments or than the room in which the animals were placed. This agrees with the findings(11) that concentration of phenylalanine or tryptophan is more important to modify the tissue serotonin content than duration of experiments.

The rats fed diet containing 3.5% L-phenylalanine did not increase excretion of phenylketones in the urine which is in agreement with the slightly elevated plasma phenylalanine values. No consistently increased excretion of urinary phenylketones was observed in rats fed 5% L-phenylalanine diet. However, phenylketones as high as 4 mg phenylpyruvic acid in the 24-hour urine sample were always observed in all animals fed 7% L-phenylalanine diet. Psychological and behavior tests of rats fed the 5 or 7% L-phenylalanine diet kept in light-dark rooms are now being correlated with plasma phenylalanine and urinary phenylpyruvic acid levels.

Summary. Experimental phenylketonuria has been produced in young rats by feeding excessive quantities of L-phenylalanine. Rats fed 7% L-phenylalanine diet showed consistently high phenylalanine values and increased excretion of urinary phenylketones. When fed in a light-dark room, rats fed 3.5%, 5%, or 7% L-phenylalanine showed reduction in liver phenylalanine hydroxylase as compared with rats fed a normal diet.

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Studies on Urinary Steroids of Men Born and Living at High Altitude.* (26930)

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Sea level residents when exposed to high altitude conditions show a variety of physiological changes(1), among them elevation of the urinary steroids(2). It is not known whether high altitude natives, who also show many adaptative responses to hypoxia, likewise have changes in adrenal function. The present study was undertaken in an attempt to answer this question.

Methods. The urinary steroids of 2 groups of young, adult males were studied: one group was formed of medical students born and living at sea level, and the other of men born 10,000 feet above sea level and having lived for more than a year in Morococha, Perú (14,900 feet). Urinary determinations of steroids were performed as follows: 1) without any medication; 2) after intravenous administration of 100 mg of cortisol (10 ml of Solu-Cortef, Upjohn) at 8 a.m.; and, 3) for 2 days in which dexamethasone was injected intravenously in a dose of 2 mg (8 a. m. and 8 p. m.). "Free" and conjugated 17,21-dihydroxy-20-ketosteroids were measured in the urine by the method of Silber and Porter(3), using ethyl acetate instead of chloroform for extraction and redissolving in ethanol prior to color reaction after evaporation to dryness(4). Beef liver B-glucuronidase (Ketodase Warner Chilcott) was used for hydrolysis. Urinary 17-ketosteroids were measured by the method of Vestergaard(5). In both methods, Allen's correction(6) was employed.

TABLE I. Urinary Excretion of 17-ketosteroids and 17,21-dihydroxy-20-ketosteroids in High Altitude and Sea Level Natives.

		17,21-dihydroxy-20-ketosteroids		
	17-ketosteroids, mg	"Free," mg	Conjugated, mg	% of conjugated
Untreated subjects—24 hr				
Sea level (27)	12.87 ± 4.09†	.52 ± .25*	5.05 ± 2.28*	90.7 ± 3.16*
High altitude (27)	11.56 ± 3.03†	.50 ± .20*	5.09 ± 1.53*	91.2 ± 2.50*
0-12 hr after inj. of 100 mg cortisol				
Sea level (10)	7.69 ± 3.66*	3.76 ± .75*	24.82 ± 5.19*	86.4 ± 3.60*
High altitude (7)	8.11 ± 1.99*	3.74 ± .81*	24.16 ± 4.51*	86.5 ± 4.20*
12-24 hr after inj. of 100 mg cortisol				
Sea level (10)	6.73 ± 2.76*	.18 ± .09*	2.71 ± .75‡	93.7 ± 1.39*
High altitude (7)	6.49 ± 2.79*	.19 ± .02*	2.21 ± .88‡	93.2 ± 1.50*
1st day of Dexamethasone admin.‖				
Sea level (10)	8.72 ± 1.84†		2.84 ± .60*	
High altitude (13)	7.31 ± 2.31†		2.67 ± .61*	
2nd day of Dexamethasone admin.				
Sea level (10)	6.19 ± 1.55*		1.37 ± .42§	
High altitude (13)	5.88 ± 2.36*		1.21 ± .54§	

Figures are means ± stand. dev. No. in parentheses indicate No. of subjects in each experiment.

* P >.50. † P <.20 >.10. ‡ P <.30 >.20. § P <.50 >.40.

‖ Only values of conjugated 17,21-dihydroxy-20-ketosteroids are given because Dexamethasone is mainly excreted in the "free" form(7).

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Results and comments. There were no differences in urinary excretion of steroids between the unmedicated sea level and high altitude groups (Table I). These results agree with the findings of San Martin *et al.*(2). The quantitative similarities in the 2 groups of urinary excretion of 17,21-dihydroxy-20-ketosteroids indicated that the yields of these catabolites were identical, without, however, giving information about relative rates of production and catabolism of cortisol and cortisone in the 2 groups. The amount of injected cortisol appearing in the urine in the following 24 hours, as Porter and Silber chromogens, was the same in high altitude and sea level groups (Table I). These results and the equivalence in percent of steroid conjugation (Table I) suggested that the catabolism was the same in both groups. The similar steroid excretions reflected, therefore, similar production of cortisol by the adrenal cortex (It is assumed here that the administered cortisol is catabolized in the same manner as the endogenous one). Dexamethasone decreased urinary excretion of steroid, but did not differentiate the sea level and high altitude groups (Table I). These findings showed that rates of production of cortisol and cortisone of both groups were identical when endogenous ACTH was inhibited; and, therefore that the similarities between both groups were not promoted by ACTH stimulation but rather depended upon the adrenal cortices themselves.

Summary. No differences were found in urinary excretion of steroids between sea level and high altitude natives. The data indicated further that the catabolism of cortisol was the same in both groups. Inhibition of endogenous ACTH by Dexamethasone did not affect urinary excretion of steroids differently in the 2 groups.

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Effect of Vitamin B_{12}, Unsaturated Fat and Hydrolyzed Glucosecycloacetate on Bile Acid Excretion in Experimental Hyperlipemia.* (26931)

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Many workers have reported that cholesterol administered to animals is metabolized and eliminated mostly as bile acids(1-3). The effect of different food fats on elimination of the bile acids has also been studied. Haust and Beveridge(4) demonstrated an increased output of bile acids in the feces of human subjects receiving corn oil in the diet as compared to those receiving butter fat. Srikantia and Gopalan(5) have recently shown that fecal output of bile acids in human subjects receiving unsaturated fat is higher than in those receiving saturated fat. Recent work of Gordon *et al.*(6) supports the view that hypocholesterolemic effect of sunflower oil is associated with an increase in fecal output of bile acids. As the unsaturated fats are known to lower the higher cholesterol levels of the serum and tissues, it is reasonable to assume that unsaturated fat may lower serum

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cholesterol levels by promoting the fecal output of bile acids.

Nath and Saikia(7) have reported that high fat (saturated) given to rabbits for a long period, induces hypercholesterolemia as indicated by elevated cholesterol levels and C/P ratio whereas unsaturated fat (linseed oil) and the same level of mixed fat (saturated and unsaturated) were found to maintain lower ester cholesterol values and C/P ratio. Vit. B_{12}, essential fatty acids, inositol and aqueous extract of hydrolyzed glucose cycloacetoacetate (GCA) also checked the rise of cholesterol levels of plasma and tissues in experimental hyperlipemia(8).

The present work was, therefore, undertaken to study the effect of hydrolyzed GCA as well as Vit. B_{12} and unsaturated fat (groundnut oil) on excretion of fecal bile acids in animals with hyperlipemia induced by feeding high saturated fat diet.

Materials and methods. Thirty male albino rats weighing an average 125 g were divided into 5 equal groups. Animals of Group I were given basal diet consisting of sucrose 16%, casein 20%, groundnut oil 20%, whole meal wheat flour 39%, salt mixture(9) 5%. Group II received saturated fat diet containing same percentage of sucrose, casein, whole meal wheat flour and salt mixture as in basal diet, but groundnut oil (20%) was replaced by Dalda[†] (20%). Group III animals were given the same diet containing mixed fat 20% (10% Dalda + 10% groundnut oil) in place of 20% Dalda. Vitamin mixture consisting of Vit. B_1 .4 mg, Vit. B_2 .4 mg, folic acid .2 mg, pyridoxine .3 mg, niacin 3 mg, calcium pantothenate 2 mg, inositol 20 mg, and codliver oil (Vit. A & D) 10-15 drops were added/100 g of the diet. The animals were supplied with food and water *ad libitum*. Animals of Groups IV and V which received high saturated fat diet as did Group II, were injected subcutaneously with hydrolyzed product of GCA (120 mg) and 5 μg Vit. B_{12}[‡] respectively/100 g body wt/day.

GCA was prepared according to the method of West(10) as modified by Nath *et al.* (11). Hydrolyzed GCA was prepared by hydrolyzing 1 g of GCA with 5 cc of 2N HCl for 20 minutes in a boiling water bath. The pH was then adjusted to 7.2 with 2N NaOH. The solution was filtered and filtrate repeatedly washed with ether. Aqueous layer was separated and traces of ether were removed. The experiment was continued for 8 weeks, after which the rats were caged separately for fecal collection. Feces were collected for 4 days and stored in cold.

Bile acids estimation. Since bile acids in feces are not in the conjugated form, the measurements here were made on free bile acids, *i.e.*, cholic, and dihydroxycholanic acids. The method for determination of these 2 bile acids was similar to that adopted by Haust and Beveridge(4) for measurement of cholic and dihydroxycholanic acids in feces.

Extraction of feces and column chromatography. Extraction procedure followed was similar to that of Carey and Watson(12) and final resulting mixtures were subjected to column partition chromatography as suggested by Mosbach *et al.*(13), using celite column which utilized 70% aq. acetic acid as the stationary phase and petroleum ether-isopropyl ether (IPE) mixture as moving phase. Elution was started with a mixture containing 40% IPE in petroleum ether and followed by gradual increase in IPE up to 60% in petroleum ether. IPE 40 and IPE 60 fractions were collected separately and evaporated to dryness and dissolved in 65% sulphuric acid (AR Grade) and heated at 60°C for 15 minutes. The 2 bile acids were determined spectrophotometrically according to the method of Mosbach *et al.*(14) by differential absorption at 320 mμ and 385 mμ for cholic and dihydroxycholanic (deoxy cholic and chenodeoxycholic acids, the 2 isomers) acids respectively with the Beckmann DU Quartz Spectrophotometer. Since IPE 40 and IPE 60 fractions behaved spectrophotometrically like dihydroxycholanic acid and cholic acid respectively, they were reported as dihydroxycholanic and cholic acids.

At the end of the feeding period, the ani-

[†] Hydrogenated groundnut oil, Hindusthan Lever Product Private, Ltd.

[‡] Macrabin, Glaxo Laboratories, India Private Ltd.

TABLE I. Values of Bile Acid Excretion in Feces and Total Plasma Cholesterol of Rats from Different Groups, with Standard Deviation of Mean Values (6 Rats/Group).

Exp. groups		Cholic acid (mg/rat/24 hr)	Dihydroxycholanic acid (mg/rat/24 hr)	Total plasma cholesterol, mg %
I	Basal diet	.530 ± .023	.553 ± .014	121 ± 12
II	Saturated fat (hydrogenated) diet	.372 ± .028	.414 ± .034	205 ± 11
III	Mixed fat (hydrogenated fat + groundnut oil) diet	.536 ± .052	.543 ± .036	174 ± 9
IV	Hydrogenated fat diet and GCA(h) inj. (120 mg/100 g body wt)	.801 ± .036	.726 ± .057	138 ± 15
V	Hydrogenated fat diet and Vit. B_{12} inj. (5 μg/100 g body wt)	.760 ± .033	.648 ± .06	152 ± 14

mals were sacrificed and total cholesterol of plasma was estimated according to the method of Schoenheimer and Sperry(15) as modified by Mookerjea and Sadhu(16).

Results. Table I shows bile acid values (cholic and dihydroxycholanic acids) of the feces of the rats of different groups. Since total bile acids were not estimated, values of 2 bile acids obtained here do not account for the total fecal bile acids. Bergstrom and Norman(17) demonstrated the modification of cholic acid to compounds containing ketonic groups in the feces. But we obtained values for cholic and dihydroxycholanic acids in feces of rats, as Haust and Beveridge reported in human feces.

The animals receiving 20% hydrogenated fat excreted less bile acids than those receiving basal diet containing unsaturated fat and mixed fat containing 10% hydrogenated fat + 10% groundnut oil. Plasma cholesterol levels of saturated fat ingested animals are higher than those of animals receiving unsaturated fat and mixed fat. Hydrolyzed GCA and Vit. B_{12} when injected to the saturated fat ingested animals show pronounced effect on excretion of fecal bile acids. Plasma cholesterol levels of the animals of Groups IV and V injected with hydrolyzed GCA and Vit. B_{12} respectively, are also much lower than those receiving hydrogenated fat.

Discussion. From the above observations, it is noted that elimination of bile acids in feces is affected by the nature of the fat. The hydrogenated fat (20%) in absence of unsaturated fat appears to have increased plasma cholesterol levels through its diminishing effect on fecal elimination of bile acids. When equal quantities of saturated and unsaturated fats are given maintaining total dietary fat at 20%, bile acid excretion is increased, accompanied by fall in total plasma cholesterol level. This effect can be attributed to the unsaturated fat.

Gordon *et al.*(6) have reported an increase of fecal bile acid elimination with a concomitant fall in serum cholesterol levels when sunflower seed oil was substituted for coconut oil in the diet. Byers and Friedman(18) have also observed that bile obtained from the common bile duct was richer in cholic acid following the feeding of unsaturated fats than following saturated fats.

The observations of other workers, and our findings support the possibility that increased excretion of the end products of cholesterol as bile acids may be one of the possible mechanisms by which substances like Vit. B_{12}, hydrolyzed GCA and unsaturated fat bring about reduction in plasma cholesterol levels. The mechanism of inhibition of bile acid excretion in animals fed hydrogenated fat can be supported by the observation of Alfin-Slater *et al.*(19) that in the absence of essential fatty acids, there is marked increase in the deposition of cholesterol esters of fatty acid which are not available for normal metabolism.

Vit. B_{12} and hydrolyzed GCA which was found to contain dienol glucose by Nath and Bhattathiry(20) may, by stimulating the cholesterol metabolism, restore the normal metabolism of cholesterol which is hampered by saturated fat. Nath and Saikia(21) have suggested that hydrolyzed GCA and Vit. B_{12} through their property of increasing the content of methionine in the liver, aid the biosynthesis of choline which results in the increase of phospholipid, and thus may prevent the rise of C/P ratio and atherosclerosis. Besides this, hydrolyzed GCA and Vit. B_{12} may be helpful in cholesterol metabolism.

Summary. The effect of Vit. B_{12}, unsaturated fat and hydrolyzed glucose cycloacetoacetate (GCA) on total plasma cholesterol levels and on excretion of fecal bile acids in rats with hyperlipemia induced by feeding saturated fat has been studied. These substances have been found to increase the output of fecal bile acids with a concomitant fall in total plasma cholesterol levels, thus indicating their important role in increasing the metabolism of cholesterol in the living system through its conversion to bile acids. Hydrolyzed GCA was found to be most effective in this process.

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Sex Differences in Maximal Hematocrit Values in the Rat in Response to $CoCl_2$ Administration.* (26932)

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The effect of gonadal hormones upon erythropoiesis has been studied in many different species and several facts are generally agreed upon. Hematocrit levels, erythrocyte counts and hemoglobin content: (A) are higher in the intact male than in the intact female; (B) fall in the male and rise in the female following castration; (C) rise in the castrated male following testosterone administration and fall in the castrated female following estrogen administration. Furthermore, Finkelstein, *et al.*(1) have shown that the male rat recovers from hemorrhagic anemia faster than the female and that androgen accelerates while estrogen delays the recovery time. More recently Mirand, *et al.*(2)

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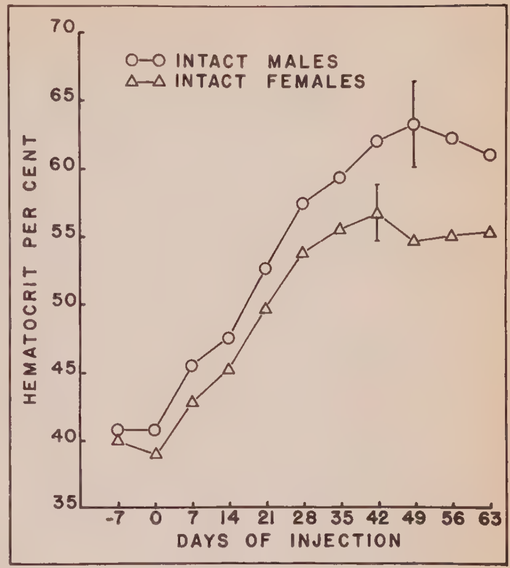
have demonstrated that uptake of Fe^{59} by the normal rat is inhibited following estrogen treatment. From these observations it might be predicted that maximal erythropoietic response to a strong erythropoietic stimulus would be greater in the male than in the female. During an experiment in which $CoCl_2$ was used to produce polycythemia we noted significant sex differences in maximal hematocrit values which to our knowledge have not been previously described.

Methods and materials. Albino rats of the Wistar strain were obtained from the Harlan Industries, Cumberland, Ind. *Experiment 1.* Ten males and 10 females were injected intraperitoneally with 10 mg $CoCl_2$ (2.5 mg elemental cobalt)/kg body wt/day for 63 consecutive days, beginning at 65-70 and 75-80 days of age respectively. *Experiment 2.* Seven males and 8 females were castrated at 63-68 days of age and after 20 days were injected with 10 mg $CoCl_2$/kg body wt/day for 63 days, exclusive of Saturdays and Sundays. Eight intact males and 7 intact females received the same treatment, beginning at 92-97 days of age.

Hematocrit determinations were made weekly on all animals. Blood was obtained by clipping the tails while the rats were under light ether anesthesia. Van Allen hematocrit tubes (1.6% aqueous sodium oxalate was used as a diluent) were employed and were spun for 30 minutes at 2750 rpm in a size 1, Model SBV (head #240), International Centrifuge.

Results and discussion. In Exp. 1 (Fig. 1) the maximal hematocrit value reached by the males (63.3%) was significantly higher ($p<.001$) than that attained by the females (56.7%). A significant difference ($p<.001$) was also observed between the intact males (66.7%) and intact females (59.5%) in Exp. 2 (Fig. 2). In contrast, hematocrit values for the castrated males and females were almost identical—64.0% and 63.7% ($0.9>p>0.7$) respectively. In both experiments maximal hematocrit value was attained after 6 weeks of treatment for the intact females and after 7 weeks for the intact males. On the other hand, maximal hematocrit values were reached at the end of the seventh week by both castrated females and males.

Although hormone replacement therapy was not carried out in castrated animals, the data strongly suggest that the maximal re-



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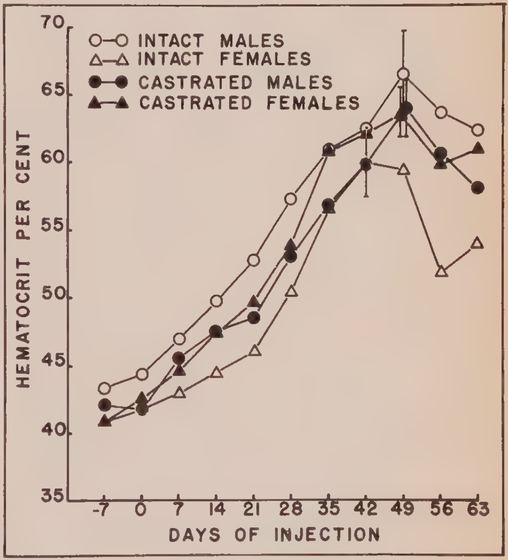

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FIG. 1. Erythropoietic response of intact male and female rats to inj. of $CoCl_2$ for 63 days. Vertical lines represent ± 1 stand. dev.

FIG. 2. Comparison of erythropoietic response of intact and castrated male and female rats following inj. of $CoCl_2$ for 63 days. Vertical lines represent ± 1 stand. dev.

sponse elicited by the strong erythropoietic stimulus of $CoCl_2$ is affected by gonadal hormones. Since the values obtained for the castrated males and females fall between those obtained for the intact males and females, the data support the conclusion of previous investigators that testosterone stimulates and estrogen inhibits erythropoiesis.

However, when the probabilities of the differences found between intact and castrated males and between intact and castrated females were calculated, p was greater than .05 but less than 0.1 for the former and less than .01 for the latter, suggesting a greater inhibitory effect of estrogen than a stimulatory effect of testosterone.

It is of further interest that an estimate of the survival time of the erythrocyte formed in response to $CoCl_2$ stimulation may be made from the data presented. It is our interpretation that maximal hematocrit level is reached when rate of erythrocyte destruction equals rate of formation; further, that this level is attained when the cells formed in response to the initial $CoCl_2$ injections begin to be removed. Accordingly, from the curves in Fig. 1 and 2 it is estimated that survival time of the erythrocytes formed in response to $CoCl_2$ is 42-49 days for the female rat and 49-56 days for the male. Burwell, *et al.* (3) employing radioiron-tagged erythrocytes established survival time of normal red cells of the rat to be 45-50 days. Therefore, estimates of survival time of erythrocytes formed in response to $CoCl_2$ stimulation, obtained from the hematocrit curves, compare quite favorably with survival times of normal cells established by more elaborate procedures.

Summary. Following injection of $CoCl_2$ for 63 days, intact male and female rats attained significantly different maximal hematocrit values of 66.7% at the end of the seventh week for the former and 59.5% at the end of the sixth week for the latter. Intermediate levels of 64.0% and 63.7% were reached by castrated males and females respectively under the same stimulus, both at the end of the seventh week. Survival time of erythrocytes formed in response to $CoCl_2$ stimulation appears to be normal.

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Fluoride Metabolism in Pregnant Rats.* (26933)

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With increasing use of fluoride salts for prevention of dental caries, metabolic and storage studies of this element (F) in humans and animals have become ever more important. Such studies in pregnant women have shown that an accumulation of F takes place in the placenta(1,2,3,4,5) and that it is also transferred to the fetus(6). At a concentration of 0.5-0.6 ppm F in the drinking water this accumulation is reflected in decreased excretion of F in urine and saliva(7). Investigations carried out in pregnant rats fed diets of varying F levels indicate that about 10 ppm of F must be ingested by the mother rat before any appreciable increase in carcass F is found in the pups(8,9,10,11,12). The present study was initiated to investigate whether F changes in the diet during pregnancy of rats would noticeably affect maternal blood, urine, osseous structure and F storage in the fetuses.

Materials and methods. One hundred female white laboratory rats of the Hebrew

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TABLE I. Fluoride Quantities Given 6 Groups of Normal and Pregnant Rats.

Group	F in food	F in water	F total
	ppm		
I	.2	0	.2
II	.2	5	5.2
III	4.55	.65	5.2
IV	4.55	10	14.55
V	4.55	50	54.55
VI	4.55	100	104.55

University strain Sabra, weighing 170 ± 20 g S. D., were divided into 6 groups receiving different F diets:

Group I was given a F-poor diet of 0.2 ppm F, consisting of 73% starch, 18% casein, 5% soya oil, 4% salt mixture, and vitamins, added as powder (thiamine HCl 0.5 mg, riboflavin 0.7 mg, pyridoxine HCl 1.0 mg, calcium pantothenate 140.0 mg, nicotinamide 7.0 mg, choline chloride 25.0 mg per 100 g of diet). Aqueous suspensions of the following vitamins were also added: Vit. A—120 u/rat/day, Vit. D—25 u/rat/day and Vit. E—0.8 mg/rat/day. Lower vitamin contents resulted in a marked degree of barrenness. The drinking water was distilled.

The rats in Group II received the same F-poor food as Group I (0.2 ppm F) with a further 5 ppm F (as NaF) in (distilled) drinking water.

In Group III, the food (mixed grain) contained 4.55 ppm F and the drinking (tap) water 0.65 ppm F.

Group IV received the same mixed grain diet (4.55 ppm F) with a further 10 ppm F in the drinking (tap) water.

Group V received the same food containing 4.55 ppm F and 50 ppm F in drinking (distilled) water.

The rats in Group VI received the same diet containing 4.55 ppm F together with a 100 ppm F level in their drinking (distilled) water.

Food and water were given to all groups *ad libitum*. The total F supply of the 6 groups is summarized in Table I.

The female rats were housed 5 to a large cage and nourished on the respective F diets for one week before the experiment, to allow the animals to adapt themselves. The male rats were introduced one into each cage and left there for 2 weeks, during which time about half the female rats could be considered to have been successfully mated. Then the female rats were put in metabolic cages thrice weekly and urine was collected during 24 hours for F examination.

The pregnant rats were separated a few days before delivery and sacrificed, together with their pups, immediately after delivery. The fetuses were weighed, homogenized, alkalinized to pH 9 and the mash dried. The femur bones, the pooled blood as well as the fetal bone tissue were examined for their F contents.

The rats which had not become pregnant during the 2 weeks' mating period served as controls.

Measuring the F content. Bones were ashed, pulverized, weighed and placed in F stills for distillation. The urine and blood samples were alkalinized with F-free $Ca(OH)_2$ in portions of 25 ml; 2 ml of a 2% solution of $Ca(OH)_2$ were added as F fixative and the whole evaporated to dryness. After ashing, the samples of urine, blood and fetus bone-mash were transferred to the F stills. F was steam-distilled at 135°C in the presence of 20 ml sulfuric acid (50%), and determined colorimetrically in the distillate, as previously described by us(5,13). The accuracy of the F determination in urine, blood and fetus bonemash was ± 0.03 ppm and in the bones ± 5%.

Results. Table II (a & b) indicates that F concentration in the femur ash of pregnant rats receiving F diets ranging from 0.2 to 104.55 ppm does not differ from F concentration in the femur ash of non-pregnant rats receiving the same F diet. By increasing F intake above a 14.55 ppm level, the F concentration in the femurs of both groups increased, but not in the same proportion as the F concentration in the diets. F concentration in the offspring of mothers receiving a low F concentration in the diet, ranging from 0.2 up to 5.2 ppm F, was almost identical. At higher F concentration in the diet, F concentration in the fetuses increased accordingly.

In fetal bone-mash we found F values

TABLE IIa. Fluoride Metabolism in Non-pregnant Rats Receiving Different Amounts of F.

		Non-pregnant rats				
		Femur, ppm F			Urine, ppm F	
Group	F supply, ppm	Range	Mean	Blood, ppm F	Range	Mean
I	.2	280-300	297	.09; .12	.02- .19	.10
II	5.2	340-360	350	—	.11- .30	.17
III	5.2	300-330	315	.10; .13	.10- .22	.14
IV	14.55	320-340	330	.12; .13	.13- .32	.23
V	54.55	530-560	540	.17	.14-1.6	.54
VI	104.55	710-740	725	.15	2.5 -3.7	3.0

TABLE IIb. Fluoride Metabolism in Pregnant Rats and Their Fetuses Receiving Different Amounts of F.

		Pregnant rats					Fetuses	
		Femur, ppm F			Urine, ppm F		Fetus bones, ppm F	
Group	F supply, ppm	Range	Mean	Blood, ppm F	Range	Mean	Range	Mean
I	.2	270-300	289	.10	.04- .27	.11	.300- .330	.317
II	5.2	300-350	329	—	.08- .26	.19	.300- .360	.330
III	5.2	305-345	319	.09	.10- .28	.16	.300- .335	.318
IV	14.55	290-320	309	—	.13- .32	.23	.425- .550	.487
V	54.55	540-550	546	—	.20-1.6	.62	2.700-3.335	3.062
VI	104.55	720-770	746	.14	2.0 -3.0	2.5	3.600-4.000	3.833

ranging from 0.3 to 4.000 ppm for the 6 F levels studied.

Mean F concentrations in the urine of pregnant and non-pregnant rats increased with F concentration in the diet. F concentration in blood increased only in rats receiving at least 54.55 ppm F in the diet. No difference was observed between F values in urine and blood of pregnant and non-pregnant rats.

Discussion. The main finding of these studies is that in rats an increase of fluoride in the diet up to values of at least 10-15 ppm is necessary in order to pass the placenta and to increase appreciably the total fluoride content in the pups. This agrees with earlier reports(9,10,11,12); Buttner(14) found that the teeth of the pups born of rats receiving 50 ppm F in drinking water during pregnancy were more acid resistant. In pregnant women, however, even small amounts pass to the fetus, which may produce anticariogenic effects in the primary teeth of their offspring (15).

The experiments with Group IV demonstrate the facility of increased F transfer through the rat placenta to the fetuses at a level of 14.55 ppm F in the food. This occurs at a level where the F content of the rat mother's blood and bones is stable (Group I-III) and only the surplus (left over after covering the special needs of the fetuses) is excreted with the urine of the mother animal. On the other hand, in pregnant women (shortly before delivery) a significant increase in blood F has been established when the F content of their drinking water was increased to 1 ppm(1,2,4,16). This points to a significant difference between the F metabolism of pregnant women and pregnant rats. In women the "non-exchangeable bone" reserve amounts to 99% and the "exchangeable bone" to 1% only(17). In the rat, which grows during most of its life span, the proportion between the 2 bone reserves is about 50:50. This is reflected in Table II (Groups I-VI) where F content of the bones increased in normal and pregnant rats at similar rates and attained very high levels with increased F supply. The F content of fetal bones reached much higher levels than that of the rat mothers, because their "exchangeable bone" amounts to 100% (cf. Group VI).

Summary. The F metabolism was compared in pregnant and non-pregnant rats of 170 g weight. In the bones, F content was similar in both groups, and when F supply exceeded 14.55 ppm, there was a pronounced increase in bone F content. In fetal bones, the increase appeared at lower levels of supply, *i.e.* 5.2-14.55 ppm. In the blood, F levels were similar in pregnant and non-pregnant rats. At intakes exceeding 14.55 ppm, there was only a slight increase in blood F in both groups. In the urine, F levels increased in both groups similarly, and rather steeply with increased F intake, thus indicating immediate removal of any F surplus with the urine in pregnant and non-pregnant rats. It is stressed that this metabolism is different from that observed in pregnant women, who tend to retain an F surplus during pregnancy.

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Effects of Insulin on Adrenaline and Noradrenaline Content of Some Rat Tissues.* (26934)

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It is well known that injection of insulin can deplete the suprarenals of adrenaline(1, 2,3). In contrast to these results, administration of insulin has been found to increase considerably the adrenaline content of the heart and liver(3).

Recently we have found that the adrenaline content of several organs is markedly increased in rats after injection of large doses of adrenaline(4). It therefore seemed possible that the increased amounts of adrenaline found in the heart and liver following insulin administration were due to accumulation of adrenaline released from the suprarenal glands. To test this hypothesis, the effects of insulin on adrenaline and noradrenaline contents of hearts and salivary glands were studied in animals from which the suprarenal medullae had been removed. Demedullation does not itself alter the catecholamine content of the organs studied(4).

Methods. Male albino rats of the Sprague-Dawley strain weighing 300 to 400 g were

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[†] Postdoctoral Fellow of Nat. Research Council of Canada.

TABLE I. Effects of Insulin on Adrenaline* and Noradrenaline* Contents of Hearts and Salivary Glands under Various Experimental Conditions.

Procedures	Hr of insulin	Hearts Adren.	Hearts Noradren.	Submaxillary glands Adren.	Submaxillary glands Noradren.	Parotid glands Adren.	Parotid glands Noradren.
Suprarenals intact	0	.05 ±.004	.55 ±.040	.11 ±.009	1.23 ±.073	.06 ±.006	1.04 ±.086
		n = 11		n = 13		n = 12	
	3	.16 (.13-.23)	.69 (.61-.83)	.24 (.20-.30)	1.47 (1.08-1.90)	.20 (.12-.36)	1.14 (1.09-1.23)
		n = 3		n = 3		n = 3	
Suprarenals demedullated 1 wk	0	.06 ±.007	.62 ±.033	.11 ±.005	1.41 ±.057	.08 ±.009	1.05 ±.069
		n = 10		n = 16		n = 16	
	3	.04 (.03-.05)	.57 (.43-.80)	.05 (.04-.07)	1.55 (1.35-1.83)	.04	1.03
		n = 3		n = 4		n = 1	
	5	.01 (.00-.02)	.61 (.53-.73)	.02 (.00-.03)	1.53 (1.45-1.62)	.00 (−.02-.01)	1.21 (1.04-1.46)
		n = 3		n = 3		n = 3	
	7	.02 (.00-.04)	.19 (.07-.28)	.02 ±.006	1.61 ±.117	.01 ±.009	1.02 ±.070
		n = 4		n = 11		n = 8	
	11	.01 (.00-.02)	.15 (.04-.27)	.02 (.00-.05)	1.42 (1.05-1.69)	.01 (−.02-.03)	1.09 (.81-1.20)
		n = 4		n = 4		n = 4	
Ecolid, 10 mg	7	.05 (.04-.05)	.55 (.42-.63)	.09 (.08-.10)	1.44 (1.21-1.60)	.06 (.04-.10)	1.13 (.87-1.35)
		n = 3		n = 3		n = 3	

* μg/g of tissue (wet wt). Mean ± S.E. or range (in parentheses). n = No. of extracts assayed.

used throughout. Bilateral adrenal demedullation was performed one week prior to administration of insulin and estimation of tissue catecholamines. Estimations of catecholamines were made according to the spectrofluorometric method described by Bertler, Carlsson and Rosengren(5). Extracts were prepared from the pooled tissues of 2 animals so that each extract contained material from 4 salivary glands or 2 hearts. For further technical details see Strömblad and Nickerson(4).

Insulin (zinc-insulin Toronto 80 I.U./ml) was given subcutaneously in a dose of 10 I.U. per 100 g body weight. No food was given, but glucose (3 ml of a 5% solution) was injected intraperitoneally when coma or convulsions developed.

Results. In the experiments of Hökfelt (3), the largest increase in adrenaline contents of the heart and liver was found 3 hours after injection of insulin. To confirm this observation under our experimental conditions, a group of rats with intact suprarenal medullae was examined 3 hours after injection of insulin. In these animals the adrenaline contents of the heart and the salivary glands were considerably increased (Table I).

In contrast, adrenaline levels of heart and salivary glands of adrenal-demedullated rats were found to be decreased at various time intervals following injection of insulin (Table I). The noradrenaline content of the salivary glands was unaltered at all of the time intervals studied, and that of the heart was decreased only 7 and 11 hours after insulin.

Injection of chlorisondamine (Ecolid) (10 mg in 1 ml 0.9% NaCl solution intraperitoneally) one-half hour before administration of insulin into adrenal-demedullated animals prevented the changes in catecholamine content otherwise found 7 hours after injection of insulin (Table I).

Discussion. The data presented above confirm the observations of Hökfelt(3) that administration of insulin to intact rats can cause an increase in the adrenaline contents of various tissues. However, the adrenaline levels were decreased consistently by insulin in animals from which the suprarenal medullae previously had been removed. These results provide strong support for the hypothesis that the increased amounts found in intact animals represent catecholamine released from the suprarenal medullae in response to the insulin-induced hypoglycemia and subsequently accumulated from the blood stream in the tissues studied.

The mechanism of action of insulin in reducing the adrenaline content of organs of suprarenal-demedullated rats is not entirely clear. However, the observation that ganglionic blockade prevents the effects of insulin suggests that these are mediated *via* the autonomic nervous system. This does not imply that all tissue storage sites for adrenaline are directly under the control of the autonomic nervous system. It is possible that a part of the effect of insulin on the adrenaline contents of tissues of suprarenal-demedullated animals is due to a flooding of the organism with noradrenaline which might displace some of the stored adrenaline. We previously have shown that injection of noradrenaline can decrease the adrenaline content of the organs studied(4).

The results presented above demonstrate that a catecholamine released from one site can be transported to and accumulated in other organs and tissues. This observation requires that events in other areas of the body, particularly in the adrenal medullae, as well as local synthesis, storage and release, be considered in interpreting the results of tissue analyses for adrenaline and noradrenaline.

Summary. Injection of insulin into intact rats increased the adrenaline contents of hearts and salivary glands. However, it decreased the adrenaline content of these tissues in animals from which the suprarenal medullae had been removed. The noradrenaline content of the heart was decreased after 7 to 11 hours of insulin action, but that of the salivary glands was unaltered. It is concluded that the increased amounts of adrenaline found in tissues of intact animals are due to the release of this catecholamine from the suprarenal medullae and its subsequent transport to and accumulation in the organs studied.

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Immunohistochemical Identification of Tissue Culture Cells. (26935)

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A major problem in tissue culture is the identification of the cells being cultured. This is important both in connection with the various cell lines now in existence and also in analysis of new cell lines derived from fresh tissue. In the case of existing cell lines there have been accidents in which cell lines have been contaminated by other propagated cultures(1,2,3,4), and passed on to other laboratories. These contaminations have been identified by chromosome morphology, immunological technics, and viral infectivity studies.

In the case of cells growing from a primary culture, where most of the cells appear to be fibroblasts, it is important to determine whether cells unique for the tissue in question are also in culture. We have used cultured muscle cells as a model system and have found it possible to show the presence of myosin-containing cells among outgrowth of fibroblasts from a muscle explant.

Methods. Preparation of antiserums: Rabbit antisera showing reactivity for human connective tissue (C.T.)(5) and rabbit anti-human myosin antisera(6) have been described. The rabbit antiserum showing reactivity with mouse connective tissue (C.T.) was prepared by injecting rabbits with the microsome fraction of a C3H mouse sarcoma in Freund's adjuvant. Microsomes were also given 3 times a week for 3 weeks by the intravenous route. The microsome fraction was prepared by the procedure outlined by Carruthers(7). Fluorescent antibody against rabbit γ globulin was prepared by conjugating fluorescein isocyanate to globulin fraction of horse anti-rabbit globulin(8). The absorption procedures with human, rat, and mouse liver sediments(9) and staining by the indirect method(10) have all been described.

Paired label staining procedure. Human C.T. reactive antiserum (globulin fraction) was labeled with tetramethylrhodamine isothiocyanate(11,12) and absorbed with mouse liver before use. The globulin fraction of C.T. reactive antiserum was labelled with fluorescein isothiocyanate(12) and absorbed with human liver before use. Tissue cultured cells fixed as described below were stained with one conjugate for 1 hr, washed 10 min. and retreated with the second conjugate for 1 hr and again washed 10 min. before observation. Since both conjugates react independently of each other, the order of addition can be reversed with little difference in results. The conjugates were not used combined, since mixing results in an additional dilution of each antibody activity. It was preferable to stain first with the fluorescein labeled antibody and subsequently with the tetramethylrhodamine labeled antibody.

Method of growing cells in tissue culture. Normal muscle cells from fetal and adult skeletal muscle of humans were explanted on sterile coverslips and overlaid with Eagle's media, containing calf serum. After 2 weeks, cultures showing good growth and containing myo-tubes by gross morphology were taken for study. The cover slips were washed in saline to remove excess proteins, fixed in ice cold acetone for 10 min., washed in distilled water and rinsed in saline before treatment with the appropriate antiserum.

Other human cells* grown from a cancer of the gall bladder, a malignant mesothelioma, and a cancer of the ovary (2 lines) were used. Mouse tissue culture cells were also used; these were 2 L lines of fibroblast grown in continuous culture (L929 and L4CZ)* and C3H muscle primary culture† explants also containing outgrowths of fibroblasts. A Gale melanoma hamster* line and a rabbit fibroblast line was also employed as control.

Staining procedure. Frozen sections of all human and mouse tissues were processed by the indirect staining procedure(5).

The tissue cultures were fixed in cold acetone and washed in saline prior to staining as described above(10).

Chromosome morphology. Since human and mouse lines can be easily distinguished by chromosome morphology, proof of the species of cells being employed with the anti-species antiserum was checked by observing the chromosomes. Explants were grown for 3-4 days and to the culture 2 γ colchicine was added per ml of media and the mixture was allowed to stand for 3.5 hrs at 37°C. The cultures were washed with Hanks' solution and treated with 25% Hanks' in distilled water for 5 minutes at 37°C. The cultures were then fixed in 60-25 methyl alcohol-glacial acetic acid, dried and stained with aceto-orcein before observation(13). Five cultures of each mouse and human were used for every chromosome morphology study.

Results. Staining properties of human and mouse connective tissue reactive antisera. The pattern of staining of rabbit antiserum reactive with connective tissue has been previously reported. This antiserum reacts with basement membrane of the human kidney tubules as well as with the glomeruli. It reacts with the central vein and sinusoids of liver and with the endomysium of skeletal muscle (Fig. 1)(6,7). It also reacts well

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* These cultures were kindly made available to us by Dr. G. Moore.

† These cultures were made available to us by Dr. J. Cairns.

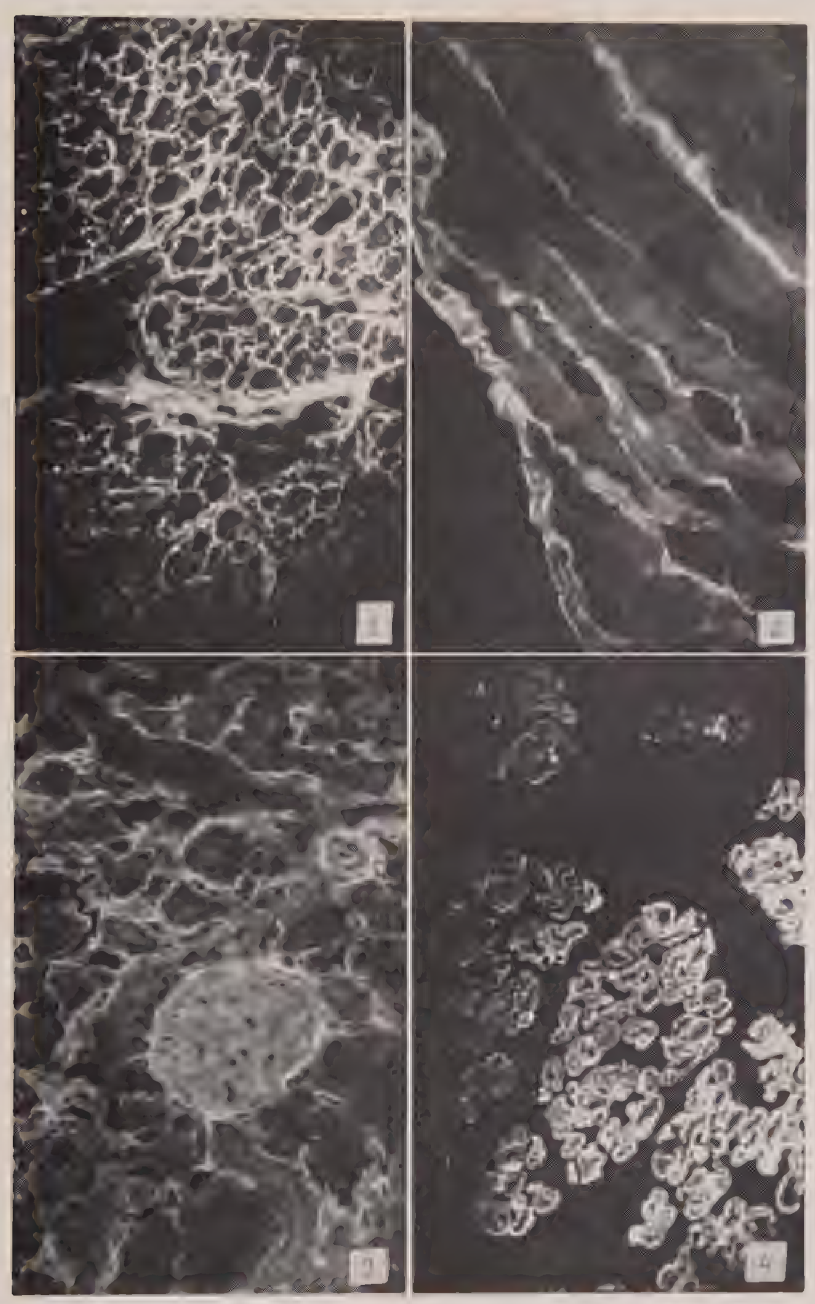


FIG. 1. Normal human skeletal muscle cross-section treated with human connective tissue reactive rabbit antiserum (absorbed with mouse liver) and subsequently stained with fluorescein labeled horse anti-rabbit globulin reagent. Staining is confined to reticular connective tissue (endomysium). No staining seen in muscle.

FIG. 2. Normal mouse skeletal muscle longitudinal section treated with rabbit anti-mouse connective tissue reactive serum (absorbed with human liver) and stained as above. Staining confined to endomysium and no staining seen in skeletal muscle.

FIG. 3. Normal mouse kidney treated as above. Staining confined to reticular connective tissue elements (basement membrane) of tubule and glomeruli.

FIG. 4. Normal human skeletal muscle cross section treated with rabbit anti-human myosin serum (adsorbed with human liver) and subsequently stained with fluorescein labeled reagent. Staining is confined to muscle elements alone. No staining seen of stroma.

TABLE I. Staining of Various Tissues (Human, Mouse, Hamster) by Several Antisera.

	Human tissue							Mouse tissue							Hamster tissue
	Organ section			Tissue culture				Organ section			Tissue culture				Tissue culture
Antiserums	Normal skeletal muscle	Normal liver	Normal kidney	Cancer of gall bladder	Cancer of ovary	Mesothelioma	Primary normal skeletal muscle	Normal skeletal muscle	Normal liver	Normal kidney	L929	L4CZ	Primary C3H fibroblast	Primary normal skeletal muscle	Melanoma
Anti-human C.T.															
Absorbed with mouse liver	C.T. only	C.T. only	C.T. only	+	+	+	+	—	—	—	—	—	±	—	—
Absorbed with rat liver	C.T. only	C.T. only	C.T. only	+	+	+	+	±	—	—	—	—	—	—	—
Anti-mouse C.T.															
Absorbed with mouse liver	—	—	—	—	—	—	—	—	—	—	±	±	—	—	—
Absorbed with human liver	—	—	—	±	±	±	±	C.T. only	C.T. only	C.T. only	+	+	+	(+)	—
Anti-human myosin															
Absorbed with human liver	+*	—	—	—	—	—	+*	+*	—	—	—	—	—	+*	—
Absorbed with mouse liver†	+	C.T. only	C.T. only	+	+	+	+	+*	—	—	—	—	±	+*	—
Chromosome morphology				human	human	human	human				mouse	mouse	mouse	mouse	

* Only muscle cells staining.

† Contains also anti-human connective tissue reactive activity that was not removed by mouse liver absorption.

—, no staining; ±, slight staining, considered essentially negative; +, staining of cellular elements.

with tissue cultured cells of human origin as determined by either fluorescent antibody or cytotoxicity(5). The human connective tissue reactive serum, when absorbed either with mouse liver or rat liver, did not react with mouse, rabbit, or hamster cells in tissue culture but did so with all the human organ section or cell lines employed (Table I). When the antiserum was absorbed with human liver sediment, all reactivity with the human tissue section and tissue culture cells was removed, as shown by lack of staining of the various tissues described above.

Rabbit antiserum prepared against mouse sarcoma reacted similarly with mouse connective tissue. The antiserum stained normal mouse organs (Fig. 2, 3) in the same area as that described for the stain of human organs by the human connective tissue reactive serum. This antiserum absorbed with human liver sediments was still capable of reacting with the mouse connective tissue antigens in organ sections as well as in tissue culture (Table I). It did not react with human, hamster, or rabbit tissue culture cells. Absorption with mouse liver sediments removed the activity quite readily.

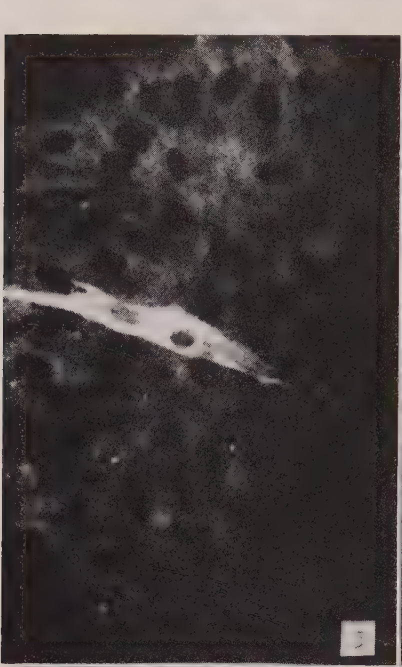
Staining properties of rabbit anti-human myosin serum. This antiserum has been shown to react with human muscle cells (Fig. 4), and also with human cells in tissue culture which contain muscle protein (Fig. 5). The antiserum after absorption with human liver sediments did not react with other human cell lines or mouse, hamster, or rabbit lines. Staining of human cells was not confined to cells that had the characteristic morphology of muscle cells in culture, but also included cells that could easily have been mistaken for fibroblasts morphologically. Cells in which longitudinal striations (myofibers) could be seen extending the length of the cell and over the nucleus were obviously derived from muscle and were strained by the antibody (Fig. 6).

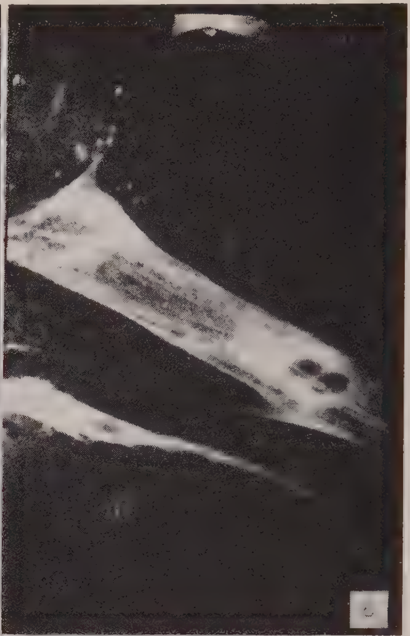
None of the tissue cultured muscle cells that were stained showed the characteristic cross-striations seen in sections of adult skeletal muscle. However these cells must have contained muscle protein because they reacted with the anti-myosin antibody. Many cells in the slide contained no detectable muscle protein since they were unstained, and were presumably connective tissue cells.

It is of interest to note that not all the cells showing the appearance of the muscle cells stained to the same extent. Some were stained more weakly than others indicating different levels of myosin or muscle protein content. This difference in staining cannot be attributed to the quality of the fluorescent label, for adjacent to these weakly staining cells were found similar appearing typical muscle cells that were stained brilliantly. This may be due to variations in the amount of muscle protein being produced by individual cells in culture and it is possible that even some of the unstained cells may be derived from muscle.

A point of interest is that the anti-human myosin serum can be used to differentiate muscle cells from non-muscle cells for other species than human due to the cross-reactions between myosins of various species. Thus the antiserum after absorption with mouse liver or human liver sediment, was still capable of combining with mouse skeletal muscle and also tissue cultured mouse muscle cells (Fig. 7, 8). This observation is probably valid for other animal muscle cultures because of the cross-reactions(14). The mouse muscle cells treated with the anti-myosin serum gave essentially the same results as that for human muscle; however, the staining was weaker.

Application of the paired label technic. By employing the paired label technic in which one antibody globulin is labeled with tetramethylrhodamine isothiocyanate and the other with fluorescein isothiocyanate, the reactions of anti-mouse and anti-human antibodies can be followed on one slide culture. It can be shown that after suitable absorption each antibody reacts with the cells of the homologous species independently of the other and no cross-inhibition occurs. When human cultures, purposely contaminated with a few mouse cells (24 hr cultures), were tested by the paired label procedure, it was easy to detect the presence of even one mouse cell in the field (Fig. 9). The anti-mouse antibody labeled with fluorescein stained the





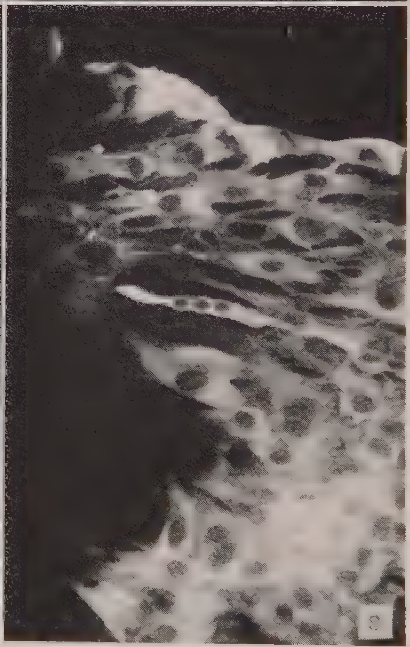


FIG. 5. Human muscle (primary explant) tissue culture treated as above. Staining confined to a single muscle cell in field. Masses of fibroblasts in background are completely negative.

FIG. 6. Human muscle (primary explant) tissue culture treated as above. Staining confined to 2 cells varying in shape and number of nuclei. Longitudinal striations can be seen fluorescing, no cross striations visible. Fibroblasts were unstained.

FIG. 7. Mouse muscle (primary explant) tissue culture treated with rabbit anti-human myosin serum (absorbed with mouse liver) and stained with fluorescein labeled reagent. A single mouse cell containing 3 nuclei is stained.

FIG. 8. Same section and field as above treated with rabbit anti-mouse connective tissue reactive serum (absorbed with human liver) and stained with fluorescein labeled antibody. Large numbers of mouse fibroblasts surrounding the single muscle cell in the field can be seen staining strongly with mouse connective tissue reactive serum.

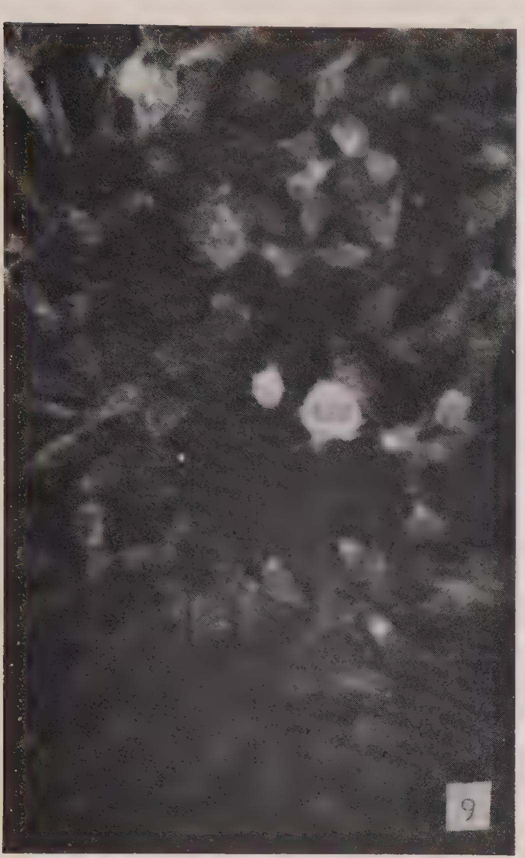


FIG. 9. Human tissue culture cells (cancer of gall bladder) purposely contaminated with a few L929 cells of mouse origin. The section was treated with fluorescein labeled anti-mouse connective tissue reactive antibody (absorbed with human liver) and subsequently with tetramethylrhodamine labeled anti-human connective tissue reactive antibody (absorbed with mouse liver). The paired labeled procedure showed 2 mouse cells which fluoresced brightly green while all background human cells fluoresced orange.

mouse cells green, whereas the anti-human antibody labeled with tetramethylrhodamine stained the human cells orange.

Summary. Cell lines of different species have been shown to be differentiated by immunohistochemical technics using properly absorbed antisera. In the work reported here the antisera were prepared against human and mouse tissues and reacted strongly with the connective tissue of the corresponding species in sections of adult human or adult mouse organs, respectively. These antisera also reacted individually with tissue culture cells of human and mouse organs. In all cases the use of the proper tissue in sufficient amounts to absorb the antiserums plays a crucial part in being able to demonstrate the specific staining of cells. Differentiation can be made even within the cell lines of a single species when unique components are present in one of them. The use of anti-myosin serum to detect muscle cells is an example. The technic of using paired labeled antiserum quickly and effectively demonstrates that very slight contamination can be recognized. Such a small contamination would be difficult to detect by other tests such as chromosome morphology technics where the cell must be in the metaphase before it can be observed and chromosomes of each cell counted.

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Failure of Ovariectomy to Influence Drum Tolerance or Phagocytic Stimulation by Zymosan in the Rat.*† (26936)

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Several observations in recent years have suggested the possibility that physiologic titers of estrogen exert a regulatory influence upon the reticuloendothelial system (RES) of female animals. However, the results have been conflicting. For example, Halpern, *et al.*(1) have shown that female mice respond to BCG infection with a greater RES stimulation than do males, but that castration of female mice does not significantly reduce the functional hypertrophy of the RES which normally follows BCG infection in unoperated females. Similarly, gonadectomy in the female rat has no effect upon phagocytic efficiency, as revealed by an unaltered rate of clearance of colloidal particles from the circulation after castration(2.) We studied the effect of ovariectomy upon stimulation of the rat's phagocytic tissues by Zymosan, an RES-stimulating agent with effects comparable to those produced by BCG. Since the development of conditioned or tolerant states in the rat appears to be associated with enhancement of RES function(3), we also studied the effect of ovariectomy upon development of resistance to rotational trauma in the Noble-Collip drum(4).

Methods. Animals. Female albino rats of the Sprague-Dawley strain (Charles River Farms), weighing between 140 g and 160 g were used. Bilateral ovariectomy was performed under light ether anesthesia.

Zymosan treatment. Suspensions of Zymosan (Lot #5B171) were prepared in sterile, isotonic saline, at a concentration of 10 mg/ml, by rapid mixing in a mortar and pestle homogenizer. Animals were injected on the fourth, sixth, and eighth postoperative days with 5 mg intravenous doses. On the tenth day after operation, and 48 hours after the last dose of Zymosan, global phagocytic index (K) was determined according to the method of Biozzi *et al.*(5). The measurement of phagocytic index was carried out with a 16 mg/100 g dose of colloidal carbon (Pelikan c 11/1431 a, Gunther Wagner, Germany) suspended in 10% gelatin and injected intravenously.

Drum tolerance. Other animals were conditioned in the Noble-Collip drum according to the following regime. Two hundred turns were given on the 4th postoperative day, 200 turns on the 5th day, 300 turns on the 6th and 7th days, and 500 turns on the 8th day. On the 9th postoperative day, the animals were rested and the next day were challenged with 900 revolutions in the drum, a normally LD/100 procedure. Weights of all animals were recorded daily throughout the training period and up to the day after challenge.

Autopsy data. Animals were sacrificed immediately after determination of phagocytic index or 24 hours after challenge in the Noble-Collip drum. At autopsy, weights of liver, spleen, pituitary, adrenals, thymus, and strips of uterus were measured. The smaller organs were trimmed of excess fat and weighed to the nearest 0.1 mg on a Roller-Smith torsion balance.

Results. 1. *Development of drum tolerance in the spayed* vs. *sham-operated female rat.* By the 11th postoperative day, significant atrophy of the uteri and hypertrophy of the thymus glands could be observed in the ovariectomized group in comparison with sham-operated controls. These effects are shown in Table I as the changes in body wt/organ wt ratios. Both of these effects on estrogen target organs confirmed the existence of a state of estrogen-deprivation in the castrated animals during the experiment. Despite this fact, there was no alteration in ability of the castrated animals to respond to the conditioning process, as shown by their

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† Zymosan was kindly provided by Dr. L. J. Old, Sloan-Kettering Inst.

‡ Postdoctoral Fellow, USPHS.

TABLE I. Effect of Conditioning in the Noble-Collip Drum on Ovariectomized and Control Rats.

Exp. group	Daily wt gain (g)*	Survival after challenge	Body wt (g)/organ wt (g) ratio* × 10^{-2}				
			1 cm uterus	Thymus	Spleen	Pituitary	Adrenals
Sham-operated (12)	2.7 ± .4	12/12	44 ± 2.6	4.3 ± .2	2.6 ± .3	178 ± 9.4	31 ± 1.9
Bilaterally ovariectomized (11)	3.1 ± .4	10/11	222 ± 20.0	2.9 ± .3	2.5 ± .1	178 ± 9.4	36 ± 2.6
			$p < .001$	$p < .01$			

* Mean ± stand. error of mean.

continued weight-gain during conditioning and their response to final challenge with a normally LD/100 dose of rotational trauma. Moreover, there was no statistically significant difference between experimental and control animals with regard to size of selected "stress organs" (spleen and the pituitary-adrenal system).

2. *Stimulation of phagocytic index with Zymosan in the spayed* vs *sham-operated female rat.* Animals were treated surgically as before, and on the 10th postoperative day autopsy revealed a significant atrophic degeneration of the uterine horns of the castrated animals (Table II). Despite this state of estrogen-deprivation, RES phagocytic function in the castrated animals was increased by Zymosan to the same extent as in controls. Table II shows the changes in both groups in global phagocytic index (K), unit phagocytic index (alpha) and relative spleen and liver weights. According to Benacerraf(2), the mean normal values for K and alpha in the rat, at the dose of carbon employed in our experiments, are 0.013 and 4.5 respectively; while ratio of body weight to combined liver and spleen weights in the untreated rat generally varies about a mode frequency of 18. Zymosan treatment in both experimental and control groups resulted in a greater than 10-fold increase in K and a significant increase in alpha. The large change in global phagocytic efficiency was associated in both groups with a significant hypertrophy of liver and spleen, especially of the latter organ which increased as much as 300% in weight.

An additional observation was made with respect to the thymus gland of the Zymosan-treated rat. The data presented in Tables I and II show that Zymosan treatment prevented the thymic hypertrophy associated with ovariectomy and produced normal-sized glands in the ovariectomized-Zymosan treated group; whereas, in sham-operated rats treated with Zymosan, there was frank atrophy of thymic structures.

Discussion. The original observation of Nicol(6,7) of an increase in number and activity of phagocytic cells of guinea pig uterine endometrium during the follicular phase of the estrous cycle prompted the suggestion that estrogenic hormones may be involved in regulation of the RES of female animals. Subsequently, it was shown that treatment of mice with large doses of synthetic or natural estrogens causes hypertrophy of liver and spleen and an accelerated rate of phago-

TABLE II. Effect of Zymosan Treatment on Ovariectomized and Control Rats.

Exp. group	Global phagocytic index (K)*	Body wt (g) /liver + spleen wt (g) (W/Wls)*	Unit phagocytic index (α)*	Body wt (g) /1 cm uterus wt (g)	Body wt (g)/ thymus wt (g)
				Ratio* × 10^{-2}	
Sham-operated (8)	.186 ±.024	13.1 ±.5	7.4 ±.5	54 ± 3.0	7.8 ± 1.4
Bilaterally ovariectomized (11)	.199 ±.015	13.0 ±.3	7.5 ±.2	172 ± 12.0	4.8 ± .3
				$p < .001$	

* Mean ± stand. error of mean.

cytosis by these organs(8-10). Synthetic estrogen (diethylstilbesterol) has a similar effect upon the RES of the rat, although estradiol does not affect phagocytosis in the rat as it does in the mouse(9). Our results clearly show that ovariectomy in the rat does not interfere with the hypertrophic response of RES organs to Zymosan. This finding is in accord with that made by Halpern in his study of phagocytic stimulation by BCG in ovariectomized mice(1). It thus seems unlikely that physiologic titers of estrogenic hormone exert a significant regulatory effect during hyperfunctional states of the RES in the mouse or rat, despite the ability of certain of these steroids to cause profound stimulation of this system when administered in greater than physiologic doses.

Benacerraf has suggested that the enhanced susceptibility of Zymosan-treated mice to bacterial endotoxin results from an exhaustion of adrenal cortical function after Zymosan(11). Assuming that this phase of adrenal cortical exhaustion follows an initial phase of adrenal cortical hypersecretion brought on by the effects of Zymosan treatment, the concomitant atrophy of thymus structures in Zymosan-treated animals can be explained on the basis of the known inhibiting action of corticosteroid hormones on this organ.

Hruza(12) has reported that castration of adult female rats does not affect their resistance to single doses of rotational trauma. In our study we were concerned with the possible effects of ovariectomy upon the ability of animals to become adapted to trauma during the course of conditioning. Our finding that gonadectomy does not interfere with development of resistance is therefore in accord with results obtained with unconditioned rats.

Summary. Bilaterally ovariectomized and sham-operated rats were compared with respect to their conditioning responses in the Noble-Collip drum, and their responses to Zymosan, a RES-stimulating agent. Estrogen-deprivation in castrated animals was confirmed by the presence of atrophic uteri and hypertrophic thymus glands. Gonadectomized animals gained weight during the conditioning process and survived final challenge as well as did controls. There was no difference in the effect of conditioning upon weights of spleen, pituitary or adrenals in the 2 groups. Zymosan treatment resulted in equivalent hypertrophy of liver and spleen, and an equivalent increase in phagocytic activity per unit of tissue in the 2 groups. It was concluded that ovariectomy does not inhibit the response of the RES of the rat to stress or to stimulation with Zymosan. It is suggested that physiologic titers of ovarian hormone are not required during the development of hyperfunctional states of the RES in female rats.

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Production of an Inhibitor Substance by DA Myxovirus. Auto-Inhibition of Plaque Formation.* (26937)

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It was reported(1) that a myxovirus, the DA virus, was detected by formation of plaques under an agar overlay but not by cytopathic effect (CPE) in parallel fluid cultures. Subsequently, it was noted that no DA virus plaques appeared in cultures inoculated with high concentrations of virus, but distinct plaques were observed in cultures inoculated with low virus concentrations. The present report is concerned with observations on this "prozone" type of reaction and the possible mechanisms involved in this phenomenon.

Materials and methods. Virus strain. DA virus, a newly isolated myxovirus, belongs to the parainfluenza group(2) but is not related to the 4 known prototypes. The isolation and characterization of this agent will be reported in detail elsewhere(3). *Cell cultures.* Rhesus monkey kidney cell monolayer cultures prepared in the usual manner, either in tubes or in 3 oz. prescription bottles, were used. All cultures were maintained in a medium containing 2% calf serum and 0.5% lactalbumin hydrolysate in Earle's salt solution. *Virus assay.* Virus infectivity titrations were determined in rhesus bottle cultures by the plaque technic(4). The hemadsorption technic described by Vogel and Shelokov(5) was also used in certain experiments. Both guinea pig and one-day old chick red blood cells were used in the hemagglutination test.

Results. The prozone reaction during serial passage of virus. Undiluted tissue culture fluids containing DA virus were used in making serial passages at weekly intervals over the last 2 years. Distinct CPE was not observed in the fluid cultures at any time. Infectivity titers of each passage level were determined by plaque titration. No plaques were evident in bottle cultures inoculated with undiluted or high concentrations of virus fluid, but clear distinct plaques were obtained in bottles inoculated with 10^{-4} and 10^{-5} dilutions of the same virus suspension. Although this "prozone" phenomenon was observed in each titration, there was no significant difference in infectivity titer end points between the 2nd and the 150th tissue culture passage levels. Fluids from later virus passages were also assayed by the hemagglutination test using chick erythrocytes, and no significant change in titer was noted, although minor variations were observed from time to time. Experiments to determine if the infectivity endpoints could be changed by serial passage of the virus in which low concentrations of DA virus were serially transferred, yielded the same results as those obtained using high concentrations of virus. On several occasions, the hemadsorption technic was also used for determining the infectivity titers as compared to the plaque method. The results were essentially the same by both methods.

Growth of DA virus in fluid cultures and under agar overlay. Growth rates of the DA virus in fluid cultures and under agar were compared, using undiluted virus suspensions as inocula (multiplicity of 0.1). After 2 hours' adsorption at 37°C, unadsorbed virus was removed and the infected cultures were washed, then divided into 2 groups. One group was overlaid with the agar-medium mixture, 10 ml per bottle, and fluid medium was added to the other group of cultures, also 10 ml per bottle. Daily samples from each group were removed from the incubator and the infected cultures with the media were frozen immediately at −70°C until assay. Prior to titration, the samples were thawed and the fluids centrifuged at 500 rpm for 10 minutes, to remove agar and cell debris. Infectivity titers of each sample were then determined by plaque titrations. Day *0* samples were taken on the day of inoculation, subsequent to addition of media. Virus multi-

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* Aided by a grant from the National Foundation.

TABLE I. Growth of DA Virus in Fluid Medium and under Agar Overlay.

Days after inoculation*	Infectivity titers (log PFU/ml) Fluid medium	 Agar overlay
0	3.6	2.1
1	5.1	4.5
2	6.3	6.3
3	6.8	6.9
4	7.0	6.1
7	7.3	6.0
8	6.8	5.7
9	6.9	4.9

* Inoculum: 5.5 log PFU/bottle culture containing 3 × 10^6 cells.

plication occurred in both agar overlaid cultures and cultures maintained with fluid medium, although cellular changes (CPE or plaques) were not visible in either group (Table I). It was also noted that the virus titers obtained from the agar overlaid cultures were somewhat lower than titers obtained in fluid cultures, and it was possible that a part of the infective virus population under agar could have been lost during the process of removing the agar in order to recover the virus.

Development of hemagglutinin and occurrence of prozone reaction. Each of a series of rhesus tube cultures containing 3 × 10^5 cells was inoculated with 4 × 10^5 PFU of the DA virus. After 2 hours of adsorption at 37°C, the unadsorbed virus was removed, and 1 ml of maintenance medium was added to each tube. All infected cultures were returned to the incubator. At hourly, and then daily intervals 2 tubes were examined for CPE, and their fluids were harvested and pooled. Half of the fluid was frozen at −70°C until assayed for infectivity, and half was tested for hemagglutinin activity. The infected cell cultures were tested for virus hemadsorption by adding 0.2 ml of a 0.5% guinea pig red blood cell suspension. Virus infectivity was determined by plaque titrations. Growth curves and appearance of hemagglutinin activity are shown in Fig. 1. New infectious virus appeared at 3-4 hours after infection, and gradually increased without evidence of cytopathic changes in the cultures for as long as 3 days. Hemadsorption was first observed at 8 hours after infection, but hemagglutinin activity could not be detected in the fluids until 48 hours, increasing in titer thereafter. Fluids obtained from the first 24 hours induced no "prozone" effect. However, when harvests collected 48 to 72 hours after infection were plated, plaque formation was inhibited in the low dilutions. It was at this time, *i.e.*, 48 hours after infection, that hemagglutinin activity was detected.

Demonstration of interfering activity of DA virus infected culture fluids treated by various physical methods. Aliquots of undiluted tissue culture fluids of DA virus were treated by 1) heat at 56°C for 60 minutes; 2) ultraviolet irradiation for 30 seconds at 250 microwatts per sq. centimeter; 3) freezing and thawing 3 times; 4) filtration using a gradocol membrane of 130 mμ (APD), which was known to hold back the infectious virus; and 5) ultracentrifugation at 30,000 rpm for 3 hours. The treated samples and the untreated controls were tested for their interfering capacity against the DA virus. The results are shown in Table II. It is evident that no significant inhibition of plaque formation occurred when the treated virus fluids and the challenge virus were combined, held at room temperature for one hour, and then added to cultures. However, if the inoculum contained high concentrations of active virus particles as in the case of the sediment (Table II) which contained 4.6 log PFU of infectious virus, inhibition of plaque formation was noted. On the other hand, if the treated virus fluids were added to the cultures first,

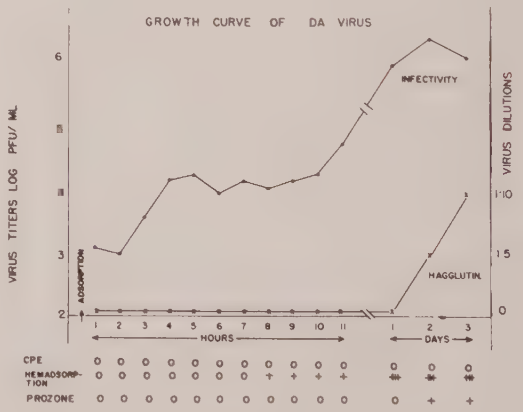

FIG. 1. Growth curve of DA virus in rhesus cell cultures, and development of hemagglutinin.

TABLE II. Interfering Activity of DA Virus Infected Tissue Culture Fluid.

Undiluted DA virus tissue culture fluid		Response to challenge with 50 PFU of infectious DA virus (avg No. plaques observed)	
Method of treatments	Infectivity titers (log PFU/ml)	Simultaneous inoculation[*]	One hr[†] adsorption before challenge
Untreated control	5.9	0	0
Treated by:			
(1) Heated 56°C	0	53	0
(2) UV irradiation	0	47[‡]	0
(3) Freezing and thawing	$<.5$	35	0
(4) Filtrate	0	18	0
(5) Ultracent. supernate	2.9	>100	0
" sediment	4.6	0	0

[*] Treated or untreated virus fluids and challenge virus were mixed and held at room temperature for 1 hr, then added to cultures simultaneously.

[†] Treated or untreated virus fluids were added to cultures and adsorbed for 1 hr at 37°C before challenge virus was added.

[‡] Minute plaques.

and adsorbed one hour before DA virus challenge, plaque formation was prevented by all the various treated materials, regardless of the presence or absence of infectious virus in the interfering system (Table II).

Discussion. The "prozone" effect of plaque formation by DA virus in the present study may be attributed to a number of mechanisms. The presence of incomplete virus(6) seems unlikely, since the infectivity titers and hemagglutinin titers of the earlier and the later passages were not significantly different when undiluted virus fluids were used for the serial transfers. The presence of non-plaque forming particles such as those observed with the influenza viruses(7) was not possible since the infectivity titers obtained either by hemadsorption or by plaque technic were essentially the same. In addition DA virus originated from single plaques(1), and was purified through several plaque passages. The "prozone" reaction was observed even when the virus was obtained directly from a single plaque and plated.

The auto-suppression of plaque formation by DA virus would seem probably to be due, at least partially, to an inhibitor substance which, in most of its properties, resembled interferon(8) and related interfering substances(9-12). There was found to be a common mechanism of such inhibition, namely, 1) protection of cells from infection by stimulation of an inhibitor using high concentration of infectious virus, and 2) protection of cells from infection by introduction of the preformed virus-free inhibitor. In the latter case an adsorption of one hour or longer prior to challenge was necessary for maximum protection of the cells.

The finding of such an inhibitor substance does much to explain some of the unusual features of DA virus replication. When heavy inocula were used, no plaques were observed, yet it was found that with the same heavy inocula virus multiplication occurred to an almost equal extent, both under agar and in a fluid medium (Table I). It appears that complete inhibition of plaque formation was not due to a complete suppression of viral multiplication, but initiation of cellular infection was dependent upon the ratio between infectious virus and inhibitor substance. Therefore, plaque formation and CPE were not observed when the concentration of inhibitor in the inocula was high enough to suppress, at least partially, virus growth.

When high dilutions of DA virus were used, small amounts, if any, of inhibitor substance may have been transferred along with the virus, but such a limited amount of inhibitor apparently was not able to prevent the initiation of an infected area. As virus multiplication continued under agar, inhibitor substance was also produced, but the newly produced inhibitor may well have diffused through the agar medium and thus had a negligible effect. Hence, in the immediate area of infection the ratio of infectious virus to virus inhibitor remained high and plaque formation could therefore continue. If, on the other hand, such an infected area was covered with fluid medium, then the inhibitor substance accumulated, and the ratio between infectious virus and inhibitor substance was lowered, probably reaching an equilibrium. In this instance, the cells were protected, at least partially, from destruction during viral multiplication, and therefore no CPE appeared.

Summary. Auto-inhibition of DA virus plaque formation was observed in tissue cultures during serial passages of this virus. An inhibitor (interferon-like) substance was found in the virus-free culture fluids. The presence of such an inhibitor substance seemed to be the determining factor for the 2 unusual phenomena observed with the DA virus: 1) plaque formation under agar overlay without CPE in parallel fluid cultures, and 2) "prozone" reaction in plaque formation.

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Effect of Chemotherapeutic Agents on Friend Virus-Induced Leukemia in Mice.* (26938)

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This study was undertaken to (a) establish methods for testing of chemotherapeutic agents against the Friend virus-induced leukemia (FVL) of mice(1) and (b) investigate the effect of a number of "established" and newer drugs.

Sugiura and Stock(2,3) have studied the effect of a variety of compounds on FVL using the spleen weight 3 weeks after virus inoculation as the criterion of evaluation. Effective compounds also were tested for their ability to increase survival time of infected animals. Mirand, Prentice, Hoffman and Grace(4) demonstrated that soon after inoculation, Friend virus produces a marked increase in uptake of Fe^{59} by the spleen. Moreover, this disease in HA/ICR Swiss mice is associated with malignant proliferation of the splenic reticulum, stimulation of the erythroid and myeloid elements of the spleen and liver, incerased hematocrit, presence of immature and mature erythrocytes in the blood, and leukocytosis(5,6).

Materials and methods. Adult mice (25-30 g), HA/ICR Swiss strain, were inoculated with the Friend virus† from a virus pool, originating from the ninth bi-weekly passage into Swiss mice in our laboratory, which was

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† Obtained from Dr. Charlotte Friend, Sloan-Kettering Inst. for Cancer Research, New York.

prepared from 10% spleen filtrates. The spleens from infected animals were harvested freshly, homogenized in a Ten Broeck grinder, and suspended to 10% by weight in cold Locke-Ringer solution. The suspension was clarified in a refrigerated centrifuge at 5000 g for 10 minutes, and the supernate filtered through a HA millipore filter. The filter was checked for ability to retain bacteria by adding *E. coli* to the suspension prior to filtration. Serial dilutions of infected and normal spleen filtrates prepared in a similar fashion were made with chilled physiologic saline. Mice were inoculated intraperitoneally with 0.2 ml of these filtrates. Daily drug administration was started 24 hours after virus inoculation and continued for 11 days. On the 12th day, part of the animals were injected intravenously with 1 μc of Fe^{59}. Iron uptake in red blood cells, spleen and liver was measured 24 hours later in a Nancy Wood well-type scintillation counter as previously described(4). Hematocrit and spleen weight measurements were made on all mice. A group of animals was followed for survival and median survival times (MST) estimated. Tests for *in vitro* antiviral effect were performed by incubating for 2 hours at 4°C dilutions of the virus filtrate with equal volumes of various concentrations of the drug under study. The drug-virus mixtures were then injected intraperitoneally into mice. Effect was evaluated as described above.

The following drugs were used: "Established" cancer chemotherapeutic agents; 5-fluoro-2′-deoxyuridine (5-FUDR), 6-mercaptopurine (6-MP), and amethopterin (methotrexate, MX). The following newer mercaptouracil derivatives, synthesized by Bardos (8,7,11) were studied: 5-mercaptouracil (AB-050)(8-10), 5, 5′ Diuracilyl disulfide (AB-051), 5-(Acetylmercapto)uracil (AB-053), 5-(Methylmercapto)uracil (AB-054). These drugs were shown to be thymine antagonists in microbiologic studies. A new antagonist of both folinic acid (but not folic acid) and riboflavin was included; 2,4-diamino-7,8-dimethyl-2,4-deoxyalloxazine (AB-200)(9-10). A series of ethylenimidophosphorocarbamates were also studied: Benzyl N-(bis-(ethylenimido)phosphoro) carbamate (AB-103), Ethyl N-(bis-(2,2-dimethylethylenimido)phosphoro)carbamate (AB-132)(12-15), and Dimethyl N-(N′, N′-bis (2-chloroethyl(amidophosphoro) carbamate (AB-155) (11). Solvents used are indicated in Table II. All drug solutions were prepared freshly before injection.

TABLE I. Relation of Spleen Weight and 24 Hour Fe^{59} Uptake to Dilution of Friend Virus Determined 14 Days after Virus Inoculation in Swiss Mice.

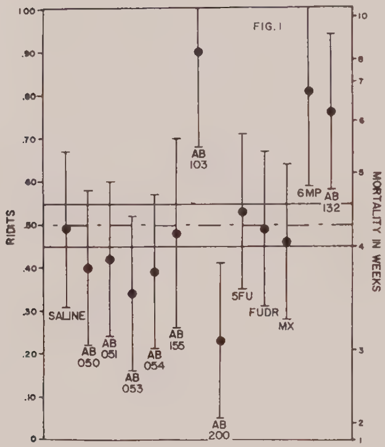
Inoculation*	Dilution of virus	Spleen wt (g)†	% of inoc. Fe^{59} taken up by spleen in 24 hr†	Hematocrit
Friend virus infected spleen filtrate	10^{-1}	1.83 ±.9	16.0 ± 3.6	47
	10^{-2}	.35 ±.02	7.6 ± 2.6	44
	10^{-3}	.27 ±.04	5.9 ± 2.3	42
	10^{-4}	.13 ±.03	3.9 ± .3	43
	10^{-5}	.16 ±.03	2.7 ± 1.0	42
	10^{-6}	.14 ±.02	2.6 ± .6	41
Normal spleen filtrate	10^{-1}	.14 ±.03	2.7 ± .04	42
None	—	.17 ±.04	2.3 ± .3	41

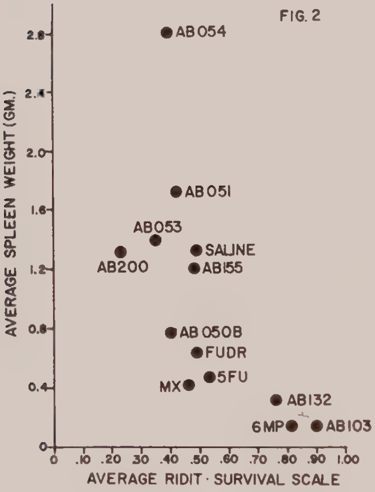
* An avg of 10 mice/dilution.

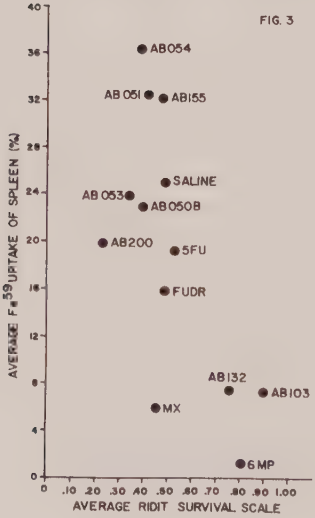
† ± S.D.

Results and discussion. Table I shows the relationship between spleen weight and 24 hour Fe^{59} uptake in the spleens of mice injected with various dilutions of FV filtrate. The splenic response closely follows the virus dilutions. Normal spleen filtrate resulted in a response similar to that of the non-injected control. Dilutions above 10^{-4} did not appear to be infectious.

Table II summarizes the effect of the various chemotherapeutic agents on spleen size, splenic uptake of Fe^{59}, and mortality. Fifteen mice were used in each group, 5 for Fe^{59} uptake and ten observed for mortality. AB-103, AB-132, 6-MP, and MX were the only drugs that caused significant decreases in spleen size and Fe^{59} uptake, compared to the control values. Only the former 3 prolonged survival time significantly as seen by analysis of the mortality data by the ridit method (Fig. 1)(16). The average ridit and 95% confidence interval is plotted for each treatment. The relationship between Fe^{59} uptake (or spleen weight) and survival on the ridit scale can be seen in the scatter diagrams of Fig. 2 and 3. It appears that when drug







treatment retains Fe^{59} uptake (or spleen weight) in the normal range (AB-103, AB-132, and 6-MP), there is an increase in survival time. MX appears to be an exception by significantly decreasing Fe^{59} uptake and spleen weight without increasing survival. On the other hand when Fe^{59} uptake (or spleen weight) becomes abnormal, there is little or no relationship to survival. There appears to be good correlation between Fe^{59} uptake and spleen weight. It is possible that these parameters are more sensitive indicators of drug effect than mortality, indicating differences between various treatments which cause no change in survival.

Experiments were undertaken to determine the effect of drugs on FV *in vitro*. Drug-virus mixtures were inoculated into mice and evaluated as in the previous treatment. At the maximal drug levels tolerated by the recipient mice, AB-103, AB-132, 6-MP and 5-FU decreased splenic Fe^{59} uptake and spleen weight, but only AB-103 and 6-MP increased survival time.

Of the drugs tested both by us and by Sugiura and Stock(3), the latter authors reported the following results: 5-FUDR (40 mg/kg/day)–negative; MX (1.5 mg/kg/day)–"slightly inhibitory"; 5-FU (35 mg/kg/day)–"moderately inhibitory", and 6-MP (30 mg/kg/day) – "markedly inhibitory". They treated the animals for only 7 days and sacrificed 3 weeks after inoculation.

Summary. 1. A variety of newer and "established" chemotherapeutic agents were tested against FVL in mice. Criteria for effectivity included spleen uptake of Fe^{59}, spleen weight, and mortality. 2. Agents demonstrating both *in vivo* and *in vitro* antiviral activity included AB-103, AB-132, and 6-MP. 3. Good correlation appears to exist between Fe^{59} uptake of the spleen and spleen weights. These 2 parameters correlate with

FIG. 1. Mortality data (analyzed by the ridit method) in HA/ICR Swiss mice infected with Friend Virus (10^{-1}).

FIG. 2. Relationship between spleen wt in g and survival (analyzed by the ridit method) in HA/ICR Swiss mice inj. with Friend Virus (10^{-1}).

FIG. 3. Relationship between 24 hr Fe^{59} uptake and survival (analyzed by the ridit method) in HA/ICR Swiss mice inj. with Friend Virus (10^{-1}).

TABLE II. *In Vivo* Effect of Drugs on Survival Time, Spleen Weights and Splenic Uptake of Fe^{59} in Friend Virus-Infected Mice (10^{-1}).

Drug	Solvent or suspending medium	Dose (mg/kg)	Route	Spleen		HCT	Mean survival time (days)
				Mean wt (g)	Mean Fe^{59} uptake (%)		
5-FU	H_2O	25	S.C.	.47 ± .24†	19.1 ± 6.74†	39.2	27.0
FUDR	H_2O	50	I.P.	.64 ± .51	15.7 ±12.04	36.0	26.0
6-MP	Saline	50	I.P.	.14 ± .02	.90± .41	30.5	40.5
Methotrexate	H_2O	1.0	I.P.	.42 ± .12	3.9 ± 2.76	31.0	25.0
AB-050B	H_2O	50	I.P.	.77 ± .23	22.8 ± 7.0	38.5	23.0
AB-051	CMC*	50	I.P.	1.72 ± .65	32.3 ± 8.1	41.2	22.5
AB-053	"	50	I.P.	1.39 ± .73	25.5 ± 9.63	46.2	23.0
AB-054	"	50	I.P.	2.01 ± .94	36.2 ±14.4	45.0	23.0
AB-200	"	25	I.P.	1.31 ± .55	19.7 ± 8.2	47.3	17.0
AB-103	H_2O	30	S.C.	.18 ± .09	7.3 ± 1.4	38.3	65.0
AB-132	Saline	100	I.P.	.31 ± .25	7.4 ± 2.3	43.5	42.0
AB-155	Warm saline	50	S.C.	1.21 ± .34	32.0 ± 8.4	54.4	19.0
Saline	—		I.P.	1.32 ± .47	24.9 ±11.2	44	26.0

* Carboxymethylcellulose 0.5%.

† ± S.D.

survival in cases where they remain in the normal range. Once they become abnormal, no quantitative relationship exists between survival on one hand, and spleen weights and Fe^{59} uptake on the other.

The authors acknowledge the invaluable assistance of Dr. I. Bross (in the statistical analysis), Mr. Gerald Goldstein (Summer Program Participant), Miss Helen Fox, Mr. Edward Dywinski, Mr. Andre Bulba, Mrs. Ruth Deckert, and Mr. A. G. Mirand. All newer compounds used were synthesized and provided by Dr. T. J. Bardos.

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Multiple Forms of Esterases from Human Erythrocytes.* (26939)

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Different forms of enzymes which hydrolyze carboxyl ester linkages (esterases) have been described from the blood plasma of man and other vertebrates(1-4), and several forms of esterases have also been reported from mouse red cell hemolysates(4). The present communication describes a system in which multiple forms of human red cell esterases can be resolved by zone electrophoresis, and presents evidence which suggests that some of the observed variations in these enzymic forms are under genetic control.

Methods. Erythrocyte hemolysates were prepared by lysing the washed, packed red cells with one volume of distilled water and extracting with 0.4 volume of toluene. Aliquots of the hemolysates were then subjected to electrophoresis in a vertical starch gel system similar to that developed by Smithies(5). Satisfactory resolution of the enzyme patterns (zymograms) during electrophoresis was produced with a bridge buffer solution (pH 8.0) containing 0.3 M H_3BO_3 and 0.05 M NaOH and a gel buffer (pH 8.6) containing 0.02 M H_3BO_3 and 0.0046 M NaOH. Electrophoresis was carried out with a gradient potential of about 7.0 volts/cm (current: 5MA/gel) for 18-20 hours at 3-5°C. After electrophoresis, the zymograms were visualized on the gels with Blue RR salt as the dye coupler and the acetate, propionate, and butyrate esters of α-naphthol as substrates. The dye-coupling procedure and inhibition study method were essentially those described by Markert and Hunter(6).

Characterization of the esterase forms. Nine bands of enzyme activity have now been resolved by this system, a composite of which is diagrammed in Fig. 1. Bands A_1 through A_8 showed the highest hydrolytic activity with the shorter acyl chain esters in the order: α-naphthyl acetate > α-naphthyl propionate > α-naphthyl butyrate, whereas the reverse was true with band B. The most consistently observable bands in their order of prominence were: A_4, B, A_7, A_8, and A_6; bands A_1, A_2, A_3 and A_5 were regularly seen only in fresh, undiluted hemolysates. Band A_8 was the slowest of the prominent esterase forms to develop with α-naphthyl acetate. Band A_7 was characteristically diffuse.

Further characterization of band B was demonstrated by the fact that its rate of anodal migration, relative to the A esterases, was increased by addition of NaCl to the bridge buffer.

It was not possible to inhibit any of the A esterases with eserine sulfate (10^{-2} M), diisopropylfluorophosphate (10^{-3} M), and B.W. 284C51j dibromide (10^{-4} M). The B esterase was inhibited by diisopropylfluorophosphate (10^{-4} M) but not eserine (10^{-2} M) or B.W. 284C51j dibromide (10^{-4} M).

On the basis of these results, the A esterases could be classified as arylesterases and the B esterase as an aliesterase according to the terminology applied by Augustinsson(1) to plasma esterases. The cholinesterase of the red blood cell could not be demonstrated by the system used in the present study.

Some diminution of enzyme activity as well as formation of methemoglobin (MetHb) was characteristic of certain hemolysates stored at −10°C. However, no effect on electrophoretic mobility of esterase bands was observed in the zymograms of these stored hemolysates.

Possible acquired variations. What appeared to be a decrease in intensity and an increase in migration rate of the A_4 and A_7 esterase components was noted in 2 cases of patients suffering from cirrhosis of the liver with associated anemia (Fig. 2:b). A similar alteration was seen in a jaundiced child with acute hemolytic anemia (reticulocyte count: 35.5%); however, the apparently altered A_4 band migrated slightly faster than in the above cases and showed no decrease in in-

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* This work was supported in part by a grant from U. S. P. H. S.

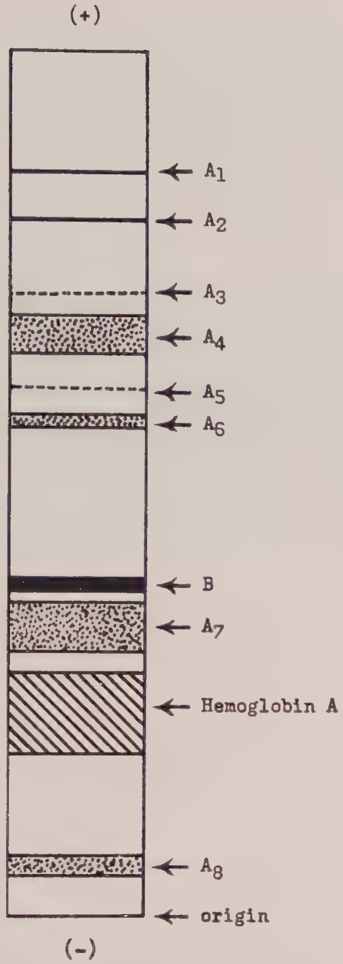

FIG. 1. Composite diagram of multiple esterase forms from normal human erythrocyte hemolysates.

tensity. When this patient was seen again the reticulocyte count had returned to 3.5%, and no alteration in bands A_4 or A_7 was observed (Fig. 2:d,e).

Clearly, these esterase forms can be affected by environmental factors which may be induced by certain clinical states. The nature of these alterations is presently under investigation.

Genetic variation. A survey of red cell hemolysates from adult, male, monozygotic twins was undertaken for the purpose of detecting phenotypic differences in esterase patterns which might be under genetic control. During this survey, one set of twins was found to show what appeared to be a decreased activity and doubling of the A_4 and A_6 components (Fig. 4: d, e). The involvement of adjacent bands suggests a structural relationship among bands A_3 through A_6.

Fresh blood samples were subsequently obtained from the mother, father, and one sister of the twin propositi. The same variation was found in the mother and the twins, while the sister and father showed the normal pattern (Fig. 4). The medical histories of the mother and twins were felt to be noncontributory.

This family is now under investigation and a complete report is forthcoming.

Summary. A system is described in which multiple bands of esterase activity can be resolved from human red cell hemolysates by zone electrophoresis in starch gel. Variations from the normal pattern were observed in certain patients with liver disorders and associated anemias. Another atypical esterase

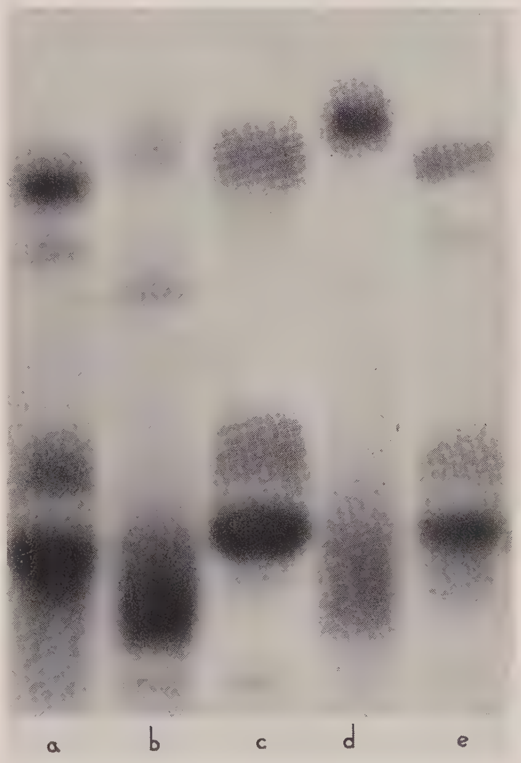

FIG. 2. Zymograms (substrate: α-naphthyl acetate) of normal and altered esterase forms from human erythrocyte hemolysates: normal subject (a); patient with cirrhosis of liver and anemia (b); individual *b* from Fig. 4 (c); patient with jaundice and hemolytic anemia (d), and same subject after treatment (e).

pattern was found in a pair of monozygotic twins and their mother indicating that the observed variation is under genetic control.

It has now been demonstrated that band A_4 is composed of two distinct bands. In the genetically determined variation the slower band of this doublet migrates at the same rate as in the normal pattern whereas the advanced band migrates faster than normal. A new band has also been located between bands A_4 and A_5 which shows decreased activity in the atypical condition under genetic control.

I am very much indebted to Drs. Margery W. Shaw, Robert L. Hunter, and Donald L. Rucknagel for prompt and valuable help and advice during this study. The excellent research assistance of Mrs. Dorothy P. Douglas is especially acknowledged.

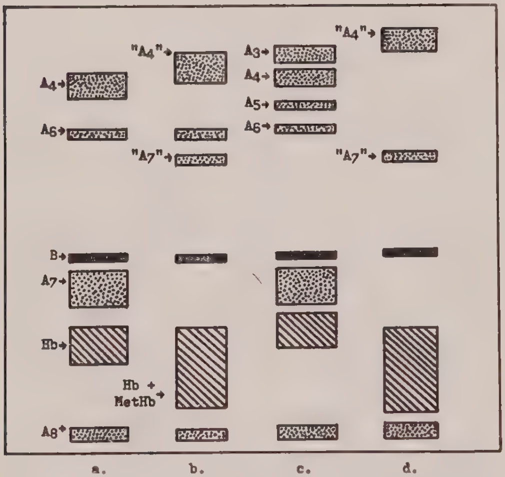

FIG. 3. Diagram of Fig. 2. Location of band *B* is indicated even though it is not well defined.

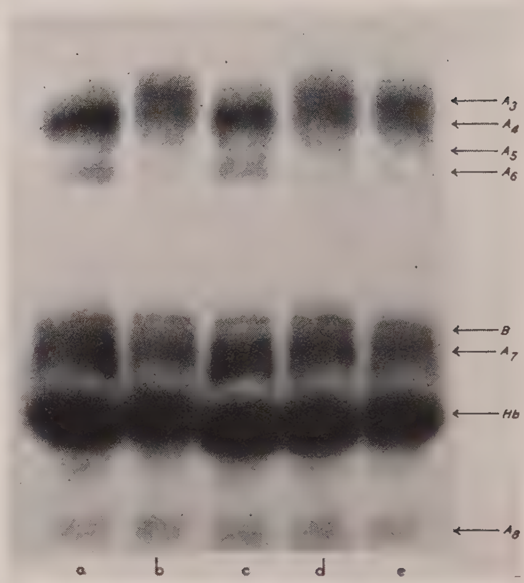

FIG. 4. Zymograms exhibiting genetic segregation of normal and atypical esterase forms from the family study: father (a); mother (b); daughter (c); and identical twin sons (d, e).

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Effects of 5-Br-Deoxyuridine and 5-F-Deoxyuridine on Nucleic Acid Metabolism by Rabbit Bone Marrow Cells.* (26940)

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A series of investigations has demonstrated that 5-bromodeoxyuridine (5-Br-deoxyuridine) may be utilized in lieu of thymidine for deoxyribonucleic acid (DNA) biosynthesis in animal and microbial systems(1-5). The anti-metabolite is not only incorporated into DNA but also blocks the incorporation of thymidine and thymidine precursors into DNA(6-10). 5-fluorodeoxyuridine (5-F-deoxyuridine) is known to inhibit formation of DNA thymine and specifically interferes with methylation of deoxyuridylic acid to thymidylic acid(6). In view of the high mitotic rate in bone marrow it seemed desirable to study the effects of these antimetabolites on nucleic acid metabolism in marrow cells.

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* Supported by research grant from U.S.P.H.S.

† U.S.P.H.S. post-sophomore research fellow.

Methods. New Zealand and California rabbits of both sexes weighing 500 to 1500 g were used. The rabbits were fed commercial chow and as prophylaxis for coccidiosis were given 0.04% sodium sulfaquinoxaline in the drinking water for 4-day periods alternating with 3-day periods of tap water.

The marrow from both tibias and femurs was suspended in Robinson's medium(11) and filtered through several layers of gauze. The final volume of the marrow suspension was adjusted to the amount needed. Two milliliters of the marrow cell suspension containing approximately 40 μg of DNAP, were incubated in 20 ml beakers with 13.5 mg of sodium succinate as an energy source, 0.05 mg of heparin to prevent clotting, labeled nucleic acid precursors, varying concentrations of 5-Br-deoxyuridine or 5-F-deoxyuridine, and other additions as indicated to a final volume of 3 ml. The radioactive nucleic acid precursors employed were: thymidine-2-C^{14}, 1.43 μcuries per μmole and sodium formate-C^{14}, 9.5 μcuries per μmole. Different batches of P^{32} (as Na_2HPO_4) of varying specific activities were used in different experiments. In the experiments with 5-Br-deoxyuridine and P^{32} unlabeled thymidine was added to a concentration of 0.05 μmole/ml. After 1 hour of incubation in air at 37°C in a Dubnoff shaker, the marrow was subjected to a combined Schneider(12) and Schmidt-Thannhauser(13) fractionation. When thymidine-2-C^{14} was used as the precursor the lipid extraction was omitted and the nucleic acids were extracted as one fraction with hot 5% trichloroacetic acid (TCA). The nucleic acid fractions were extracted with ether to remove the TCA and aliquots of the various fractions were counted in an end-window Geiger counter.

DNA P and RNA P concentrations were determined by the diphenylamine and orcinol procedures, respectively(12).

TABLE I. Effects of 5-Br-deoxyuridine and 5-F-deoxyuridine on Thymidine-2-C^{14} Incorporation into Marrow DNA. The concentration of thymidine-2-C^{14} was 0.16 μmole/ml.

Exp. No.	5-Br-deoxyuridine, μmoles/ml	5-F-deoxyuridine, μmoles/ml	cpm/μg DNA P	Inhibition, %
1	0	0	5.7	—
	.16	0	3.6	37
	.48	0	1.9	67
	.96	0	1.2	79
2	0	0	3.5	—
	0	.16	3.4	3
	0	.48	2.9	17
	0	.96	2.3	34

Results and discussion. 5-Br-deoxyuridine greatly inhibited the incorporation of thymidine-2-C^{14} into bone marrow DNA while 5-F-deoxyuridine was much less inhibitory (Table I). Degree of inhibition exhibited by 5-Br-deoxyuridine increased linearly with concentration. A 50% inhibition was obtained with a 5-Br-deoxyuridine/thymidine concentration ratio of 1.6. To determine if the inhibition was competitive the effect of 5-Br-deoxyuridine at varying concentrations of thymidine on the Lineweaver-Burk(14) plot was determined. The results (Fig. 1), demonstrate that 5-Br-deoxyuridine acts as a competitive inhibitor of incorporation of thymidine into the DNA of bone marrow cells.

The data in Table II show that 5-Br-deoxyuridine does not inhibit incorporation of

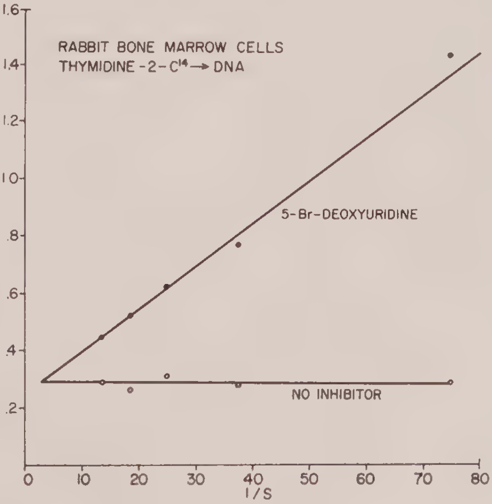

FIG. 1. Lineweaver-Burk plot(14) of inhibition by 5-Br-deoxyuridine of thymidine-2-C^{14} incorporation into bone marrow DNA. Concentration of 5-Br-deoxyuridine was .053 μmole/ml. S = μmoles thymidine-2-C^{14}/ml. V = cpm/μg DNA P

TABLE II. Effects of 5-Br-deoxyuridine and 5-F-deoxyuridine on the Incorporation of Formate-C^{14} and P^{32} into Marrow Nucleic Acids. The concentration of formate-C^{14} was 0.77 μmole per ml.

Radioactive precursor	Exp. No.	5-Br-deoxyuridine, μmoles/ml	5-F-deoxyuridine, μmoles/ml	Nucleic acid specific activity, cpm/μmole P	
				DNA	RNA
Formate-C^{14}	1	0	0	7.9	10.2
"	1	.77	0	.7	11.4
"	1	0	.77	1.1	11.6
P^{32}	2	0	0	1.8	8.7
"	2	.32	0	2.3	14.4
"	3	0	0	31.8	129.0
"	3	0	.37	27.0	92.7

phosphate into bone marrow DNA. The competitive inhibition of thymidine incorporation by 5-Br-deoxyuridine without concurrent reduction in phosphorylation indicates that 5-Br-deoxyuridine does not inhibit thymidine kinase and that the anti-metabolite is used in lieu of thymidine for DNA biosynthesis by bone marrow cells.

5-F-deoxyuridine inhibited phosphate incorporation into DNA to the same degree that it inhibited thymidine-2-C^{14} (Table II).

When formate-C^{14} was used as the DNA precursor (Table II), specific activities of DNA were drastically reduced by both 5-Br-deoxyuridine and 5-F-deoxyuridine, but specific activity of RNA was slightly increased. This indicates a specific effect of 5-F-deoxyuridine on formate utilization for thymidine synthesis and reflects the inhibition by 5-Br-deoxyuridine of thymidine utilization.

The results of these experiments demonstrate that in bone marrow cells 5-Br-deoxyuridine is incorporated into DNA in lieu of thymidine while 5-F-deoxyuridine inhibits thymidine formation. Such an effect may be related to the relative sizes of the fluorine and bromine atoms. It has been pointed out that the atomic radius of the fluorine atom is quite similar to that of hydrogen(6) while the atomic radius of the bromine atom is similar to that of the methyl group(15). Thus fluorine substituted uracil nucleosides might be expected to act as deoxyuridine inhibitors while the corresponding bromine substituted analog would act as a thymidine inhibitor.

Summary. The effects of 5-Br-deoxyuridine and 5-F-deoxyuridine on thymidine-2-C^{14}, formate-C^{14}, and P^{32} incorporation into rabbit marrow DNA *in vitro* were studied. 5-Br-deoxyuridine competitively inhibits incorporation of thymidine-2-C^{14} into rabbit marrow DNA, and is apparently used in lieu of thymidine for DNA biosynthesis. The bone marrow is quite sensitive to the action of 5-Br-deoxyuridine. 5-F-deoxyuridine does not appreciably affect incorporation of thymidine-2-C^{14} or P^{32} into marrow DNA or formate-C^{14} into RNA but greatly inhibits utilization of labeled formate for DNA synthesis indicating an interference with thymidine formation.

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Thromboplastic and Fibrinolytic Activities of Large Human Vessels.* (26941)

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Intima and media of the human aorta are highly thromboplastic with little or no fibrinolytic activity, while adventitia is less thromboplastic and highly fibrinolytic(1). Hence, after tissue injury fibrin can be easily deposited on the intimal surface. Its removal, however, depends upon a fibrinolytically active system in blood. We have now investigated some large human vessels to elucidate further the physiological role of the hemostatic balance in the organism.

Experimental. Materials and methods were similar to those previously described (1). Thromboplastic activity was estimated on freshly drawn citrated rabbit plasma and compared with a suspension of human brain used for prothrombin estimations. Fibrinolytic activity was estimated after extraction with KSCN and expressed in units of a standard preparation of plasminogen activator prepared from pig heart. The fibrin plate method with 0.12% bovine fibrinogen clotted with bovine thrombin (Leo Pharmaceutical Prod., Copenhagen) was used. All estimations (thromboplastic and fibrinolytic) were made on serial dilutions of the samples.

Samples of the vessels were obtained from autopsies (21) at the Inst. of Forensic Medicine, Univ. of Copenhagen (with kind permission of Prof. H. Gormsen). Most of the samples came from subjects who had died from accidents. As in our previous report apparently normal or only slightly pathological specimens were compared. Complete separation of layers (under water) was obtained for arteries only. In veins media and adventitia could usually not be separated and the activities had to be estimated together. No effort was made to separate the endothelium from the intima proper, since nearly identical activities had been found in our previous study.

Results. The thromboplastic activity in the different layers of the vessels appears in Table I. In all vessels thromboplastic activity was low in adventitia. In the pulmonary vein and inferior vena cava it was also low in the intima. It reached medium values in the media and intima of the pulmonary artery. As before aortic intima was nearly always highly active. None of the curves indicated presence of antithromboplastic material. The activities of aortic and coronary intima in the same individuals were estimated in 5 subjects (Table II). The distribution follows identical patterns and the coronary intima has even higher thromboplastic activity than the aortic intima.

Estimations of plasminogen activator concentrations in 14 subjects are presented in Table III. Fibrinolytic activity was found in the aortic intima in only 4 cases, and in only one was there more than a trace of activity. It is perhaps significant that the sample with highest intimal activity (44.4

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TABLE I. Thromboplastic Activities in Layers of Large Human Vessels (Expressed as Fractions of Activity of a Human Brain Preparation).

Age & sex		Cause of death	Aorta*			Pulm. artery			Inf. vena cava		Pulmonary vein	
			Int.	Med.	Adv.	Int.	Med.	Adv.	Int.	Med. + Adv.	Int.	Med. + Adv.
8	♀	Cerebellar haemorrhage	1/21	—	<1/128	1/64	1/16	<1/128	1/100	<1/128	1/120	<1/128
35	♂	Accident	2/3	—	—	1/6	—	—	1/14	—	1/14	—
35	♂	CO poisoning	1/2	1/2	<1/128	1/16	1/8	<1/128	1/64	—	—	—
36	♀	Uremia	1/2	1/4	<1/128	1/12	1/15	<1/128	<1/128	<1/128	<1/128	<1/128
38	♂	Accident	1/4	—	—	1/32	1/16	1/64	—	—	1/50	<1/128
38	♂	Pulm. emb.	1/6	1/3	<1/128	1/6	1/3	<1/128	1/32	<1/128	<1/128	<1/128
38	♂	Uremia	1/6	1/12	1/32	—	—	—	—	—	—	—
41	♀	Accident	1/16	1/28	<1/128	1/40	—	—	<1/128	<1/128	<1/128	<1/128
45	♀	Spinal cord cancer	1/2	1/12	<1/128	1/10	1/14	<1/128	1/50	<1/128	1/40	<1/128
63	♂	Coronary thrombosis	1/5	3/4	1/3	—	—	—	—	—	—	—
64	♀	Accident	1/8	—	—	1/80	—	<1/128	<1/128	—	<1/128	—
70	♀	Cerebral arteriosclerosis	1/10	1/20	<1/128	1/40	1/100	<1/128	—	—	—	—
73	♂	Accident	1/4	1/8	<1/128	1/64	1/25	<1/128	1/80	<1/128	1/100	<1/128
		Avg	1/4	1/4	<1/128	1/16	1/10	<1/128	1/50	<1/128	1/60	<1/128

* Int. = intima; Med. = media; Adv. = adventitia.

units) was from a case of CO-poisoning since the only sample with measurable activity in our previous study (Ref. 1, Table III, 35 units) was also CO-poisoned. Aortic media showed trace activity in only one case. In 5 cases intima of the pulmonary artery had no fibrinolytic activity. The remaining 9 samples showed low activity. Media of the pulmonary artery was always active and was high in a single sample. Adventitia always had medium or high activity. All layers of the inferior vena cava and pulmonary vein were fibrinolytically active. Samples of aorta and coronary artery from the same individuals are compared in Table IV. The most interesting finding is that all 3 layers

TABLE II. Thromboplastic Activities of Intimal Layer of Coronary Arteries and Aortas of 5 Individuals. (Recorded as in Table I.)

Case No.	Aorta	Coronary artery
18	2/3	1/1
21	2/5	3/5
22	1/12	1/4
23	1/12	1/4
24	1/5	1/2
Avg	1/3	1/2

of the coronary artery have fibrinolytic activity, though intima is only slightly active.

Discussion. The potential ability of the arterial wall to form and dispose of fibrin deposits is of interest in study of the pathogenesis of arteriosclerosis, since fibrin formation on the inner arterial wall is the basis of the thrombogenic theory. Hence, several investigations on thromboplastic and fibrinolytic activity of arteries and veins have appeared recently.

Witte(2) observed high thromboplastic activities in the human arterial wall, while veins had lower activity. Kirk(3) confirmed the high thromboplastic activity of the human arterial intima. He found high activity in intima of vena cava, while intima from the pulmonary artery was lower. Stevenson *et al.* (4), using a different technic, found little thromboplastic activity in aortic intima, though it could substitute for platelets in the thromboplastin generation test. Todd(5), using a histological method, found fibrinolytic activity to be related to veins, while systemic arteries were inactive. In lung, fibrinolysis was produced by the pulmonary arteries and arterioles. In large arteries the vasa vasorum were active. Lieberman and Kellogg(6) confirmed the absence of plasminogen activator from arterial intima and its high concentration in adventitia.

The observation by these authors and the results of the present investigation show general agreement, though there are discrepancies. In contrast to Kirk(3) we found only

TABLE IV. Plasminogen Activator Concentration in Coronary Arteries and Aortas of 5 Individuals. (Recorded as in Table III.)

	Aorta			Coronary artery		
Case No.	Int.	Med.	Adv.	Int.	Med.	Adv.
15	0	0	456	13	48	810
20	10	3.8	432	18	27	918
21	6.2	2.7	280	19	36	583
23	0	0	385	0	5.3	172
24	0	0	296	11	29	324
Avg	3.2	1.3	370	12	29	561

TABLE III. Plasminogen Activator Concentration in Layers of Large Human Vessels (Expressed as Units/g Fresh Tissue).

			Aorta			Pulm. artery			Inf. vena cava			Pulm. vein	
Age & sex		Cause of death	Int.	Med.	Adv.	Int.	Med.	Adv.	Int.	Med.	Adv.	Int.	Med. + Adv.
4/12	♀	Accident	0	0	264	0	—	480	528	324		—	—
8/12	♀	Pneumonia	0	0	216	14	50	264	360	300	360	252	72
1	♂	Accident	0	0	552	26	122	480	240	264	1080	—	—
8	♀	Cerebellar haemorrhage	0	0	420	0	72	552	732	420	420	264	312
35	♂	CO poisoning	44	0	576	67	324	576	264	672		264	744
35	♀	Pneumonia	0	0	97	0	35	64	75	345		48	75
36	♀	Uremia	0	0	88	12.5	36	132	114	480		93	149
38	♂	"	0	0	168	3	17	138	210	132	432	96	60
45	♀	Spinal cord cancer	4	0	240	7.4	50	240	108	126	396	19	72
45	♂	Myocardial infarction	3.8	0	122	6.7	37	276	330	144	504	264	264
63	♂	Coronary thrombosis	0	0	194	0	72	252	218	—	336	84	273
64	♀	Accident	9.4	0	610	34	54	430	159	124	129	86	215
70	♀	Cerebral arteriosclerosis	0	0	324	0	37	204	—	—	—	—	—
73	♂	Accident	0	3.8	302	8.1	15	270	65	140	140	32	102
		Avg	4.4	.3	298	12.8	71	311	262	206	422	137	213

low thromboplastic activity in intima of vena cava. Though we observed fibrinolytic activity in most of the samples of pulmonary arterial intima (9 of 14) intimal activity in most samples of pulmonary vein was considerably higher and none was zero. The discrepancy between this finding and the observation by Todd(5) could possibly be due to the fact that he investigated small arteries and veins present in sections of lung tissue. We could confirm with our extraction method his histological finding of fibrinolytic activity in the intima of large systemic veins (vena cava inferior), which in some cases was even higher than in the corresponding sample of adventitia.

Summary. Thromboplastic and fibrinolytic activity was estimated in layers of large human vessels. Arterial intima has higher thromboplastic and lower plasminogen activator concentrations (usually zero) than the intima of large systemic veins (vena cava inf.). In all vessels adventitia has high fibrinolytic activity.

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Effects of Selective Ultraviolet Irradiation of the Nuclei of Living Cells.* (26942)

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The authors have reported some of the manifestations of ultraviolet irradiation damage in living cells(1,2,3). The effects reported concerned the nature of the response of the whole cell to ultraviolet irradiation, and the effects of selective ultraviolet irradiation of the cytoplasm of living cells. The purpose of this report is to extend the previous observations by recording an additional type of ultraviolet irradiation of a selected area of the cell. This report deals with the nature of the damage produced in intact living cells by selective ultraviolet irradiation of the nuclei of these cells.

Methods. We have previously reported the technic of ultraviolet flying spot television microscopy and time-lapse cinematography (4,5,6). Recently the authors have recorded improvements in this technic which permit the use of double flying spot microscopy illumination(7). In the experiments reported here the principle of double flying spot illumination was employed to illuminate the nucleus of the cell with light of a peak wave length of 2600 Å and to illuminate the cytoplasm of the same cell with light of a peak wave length of 4500 Å. Control cells in the same field were illuminated solely with light of 4500 Å wave length. Although it was impossible to irradiate the nuclei of the cells without including a small amount of cytoplasm in the irradiated area, it is felt by the authors that this amount of irradiated cytoplasm was so small in comparison to that which was protected from the irradiation that its effects were negligible.

No presently available technic could be applied to determine the dose of irradiation given to the nucleus of the cell.

The ultraviolet emitting scanner tube and the visible light emitting scanner tube—which in this case acted as the source of il-

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lumination—were permitted to operate continuously at a scan rate of 4 frames per second. Every 7 seconds the monitor tube was selectively blanked so that a new 16 mm film frame could be pulled into position. Each 16 mm film frame stored the image information from 28 scanned frames of the monitor tube. By storing the monitor tube information from multiple scan frames on one 16 mm film frame the signal to noise ratio was greatly increased. At the end of the experiments the equipment was altered so that a complete ultraviolet absorption image of all cells was obtained. This ultraviolet absorption image was obtained and recorded on the film for several minutes.

The cell type used in this experiment was Chang Liver. All of the cells were carried in stock cultures in Eagle's media enriched with horse serum and for purposes of the experiment they were transferred to Rose chambers with quartz coverslips. During the course of the experiment only, the chambers were supplied with Eagle's media without horse serum. Horse serum was removed from the media during the experiment because its absorption of ultraviolet light at these wave lengths obscured the nature of the absorption image of the cells.

In all, these results represent data taken from 5,000 feet of 16 mm black and white film. A total of 61 cells was studied.

Results. Continuous ultraviolet irradiation of the nuclei of living cells for 14 hours or more with a beam current of 25 microamps applied to the ultraviolet emitting cathode ray scanner tube produces a variety of types of damages to the cell. These effects may be divided into nuclear and cytoplasmic types.

The morphologic aspects of the nuclear effects of nuclear ultraviolet irradiation are not pronounced. The nuclear membrane often becomes more prominent in the ultraviolet field, due probably to the condensation of ultraviolet absorbing material or particulate material around it. This effect is often attended by a slight increase in ultraviolet absorption by the nucleus. These changes are observed at the end of approximately 8 hours of irradiation.

The nucleoli do not change in appearance or in amount of ultraviolet absorption during the experiment.

The cytoplasmic changes which accompany nuclear irradiation consist first of a gelling of the cytoplasm which occurs after an average of 2 hours' irradiation. This gelling is evidenced by a stasis of lipoprotein particles in the cytoplasm coupled with a cessation of pinocytosis.

After about 4 hours' irradiation, a gradual decrease in cytoplasmic area occurs, extending over several hours. The most striking evidence of the decrease in area is a thinning and withdrawal of cytoplasmic streamers as the cell attains a more nearly spherical shape.

Bubbling of the cytoplasm occurs in less than 10% of the cells during nuclear irradiation. Three different types of bubbling are seen. Our type is a large nonabsorbing bubble forming by syneresis on or from the cytoplasmic membrane. A second type differs from the first in that the bubbles are filled with cytoplasm and the contents of these bubbles exhibit ultraviolet absorption comparable to the remaining cytoplasm. Still a third type is the intracytoplasmic bubble. This type of bubble is usually multiple and is always transparent to ultraviolet light. All 3 bubbles have been observed to rupture and release their contents into the environs. One or more of these types of bubbles are observed in different cells; however, all 3 types are never seen in the same cell. When bubbling occurs, it is usually after prolonged periods of nuclear irradiation, after 14 hours or more.

Ultraviolet absorption images of the entire cell after several hours of nuclear irradiation reveal no decrease in ultraviolet absorption of the cytoplasm as compared to unirradiated cells in the field.

Two additional effects of nuclear irradiation may be noted: 1) In 10% of the experiments, free cells which happen to be in the vicinity of the cell which was undergoing nuclear irradiation tend to migrate toward, and coalesce around, the latter cell. 2) In none of the instances observed did a cell undergoing nuclear irradiation go into mitosis.

Discussion. As previously reported(2) ultraviolet irradiation applied to the whole cell by these technics may take several patterns, briefly summarized as follows. The most acute pattern is a sudden collapse of the cell into a round ultraviolet opaque ball. With less intense doses, the cytoplasm first begins to gel, then may display a variety of bubbles and lose its characteristic absorption. Coupled with these latter phenomena the nuclear membrane increases in prominence as an ultraviolet absorbing structure and begins to wrinkle as the nucleus collapses. During this period the nucleus itself begins to decrease in size and increase in absorption and may finally decrease in diameter by as much as 50% and incerase in absorption by as much as 4-fold. During these processes of cellular damage, the nucleoli do not change their size, shape or absorption.

As previously reported(3), nuclear protection from ultraviolet irradiation during constant ultraviolet irradiation of the cytoplasm prevents the nuclear manifestations of ultraviolet induced cellular damage, while conferring no degree or type of protection on the cytoplasm or on the longevity of the cell.

The cytoplasmic effects of nuclear irradiation resemble, in a lesser degree, those effects elicited by irradiation of the entire cell. The gelling of the cytoplasm is quite similar in both cases; however, bubbling of the cytoplasm is much more consistent in both frequency and type during irradiation of the entire cell than during irradiation of the nucleus alone. The sudden collapse of the cell which occurs when the entire cell is irradiated does not occur during nuclear irradiation; rather the cytoplasm withdraws gradually, and never collapses completely.

Nuclear effects of nuclear irradiation resemble those changes which occur during irradiation of the entire cell, but to a lesser degree. The most striking difference is that during nuclear irradiation marked shrinkage and collapse of the nuclei do not occur. The increased prominence of the nuclear membrane is less during nuclear irradiation and the increase in ultraviolet absorption by the nucleus is not so marked.

During nuclear irradiation, the nucleoli are not observed to change in size, shape, number, or ultraviolet absorption. In a previous experiment in which the nucleoli alone were irradiated, the nucleoli were observed to lose 90% of their characteristic absorption after 6 to 8 hours of ultraviolet irradiation(8). This loss of absorption is presumably due to an alteration of the protein and nucleic acid composition of the nucleolus; this alteration apparently does not occur when the entire nucleus is irradiated.

Previous experiments from the literature involving nuclear irradiation have differed markedly from the above experiments. The most closely analogous experiment dealt with the effects of ultraviolet irradiation of the nuclei of living amoeba(9). In this experiment, it was found that when nuclei of irradiated cells are transferred to unirradiated cells, not only is the cytoplasm of the unirradiated cell unable to reverse the damage to the irradiated nucleus, but the unirradiated cytoplasm itself is damaged by contact with the irradiated nucleus.

From the results of a similar experiment on isolated thymus and liver nuclei(10), it was concluded that cytoplasmic recovery from x- and uv-irradiation can occur with intact nuclei, with the reverse being much less obvious. The cytoplasm was not seen to be more radioresistant than the nucleus, however.

Other experiments(11,12) have dealt with the effects of uv-irradiation on cells in various stages of mitosis. It is reported that chromosomal aberrations have been induced as well as a cessation of normal mitotic progression.

Other experiments(13,14) deal with the effects of x-rays and alpha particles on DNA synthesis and mitosis in ova and other cells. It is reported(14) that a temporary decrease in DNA synthesis occurs in such cells, followed by a resumption of DNA synthesis in which the cells do not divide and giant cells are produced. Apparently, these effects are due to the irradiation of the nucleoli of the cells rather than the nuclear sap itself(14).

The reported experiments differ so greatly from the above experiment that no comparison of results is possible.

Conclusions. (1) Continuous ultraviolet irradiation of the nucleus of a living cell produces nuclear damage. Although this damage resembles nuclear damage which occurs when the entire cell is irradiated, it is less severe due to the protection of the cytoplasm from the effects of irradiation. (2) Continuous ultraviolet irradiation of the nucleus of a living cell produces cytoplasmic damage resembling that which occurs when the entire cell is irradiated, but is less pronounced.

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Septal and Anterior Hypothalamic Lesions: Some Effects on Sleep and Body Metabolism in Cats.* (26943)

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It is currently hypothesized that the posterior hypothalamus and ascending reticular formation are concerned with the state of wakefulness. In accordance with this, it has been postulated that somnolence is due to periods of depressed activity within these regions. Some authors, although supporting the relationship of these areas to wakefulness, have preferred to regard these periods of depressed function as due to influences from specific mechanisms which may be subject to extrinsic rather than intrinsic factors.

Ranson and Ingram(1), and again Ingram *et al.*(2) reported that lesions placed in a number of areas of the cat's hypothalamus caused somnolence, but no sleeplessness was noted. Kabat *et al.*(3), stimulated the preoptic region of cats without producing sleep. Ranson and Magoun(4) partly destroyed this latter region (in cats) and observed no change in sleep-wakefulness cycles. Insomnia was not mentioned by Ranson(5) after lesions in the hypothalamus of monkeys.

Nauta(6) described a condition of sleeplessness produced by elimination of the suprachiasmatic and preoptic areas in rats. Postoperatively these animals behaved more or less normally, but would not sleep and after a few days lapsed into a state of exhaustion and died. Nauta concluded that there might be an area in the preoptic region which served as a "sleep center", as opposed to a "waking center", referring to a region in the posterior hypothalamus around the mammillary nuclei. This latter area, when removed, would produce somnolence, and Nauta believed that the "sleep center" functionally inhibited the "waking center" to produce a state of sleep. Heath(7) placed lesions somewhat rostral to those of Nauta, but including some areas in common, and no sleeplessness was reported.

Considering Nauta's findings from the standpoint of metabolic and hypothalamic re-

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lationships one wonders if disturbances of metabolism could have been induced and contributed to a truly sleepless state.

This work concerns an attempt to reproduce Nauta's results in cats, and to uncover any gross metabolic disturbances that might be related to it.

Methods. Bilateral electrolytic lesions were placed by stereotaxic means in the suprachiasmatic and preoptic region of 24 cats. These were designed to conform as closely as possible to the area which Nauta incised surgically in rats.

Criteria for determining alterations in sleep and wakefulness cycles in cats present serious difficulties since each animal has its own particular pattern. Two approaches to the problem were used. One method was the observer's subjective impression. Evidence of sleep was judged on the basis of posture, position of the head, eyes open or closed, and respiratory depth and rate. A second method involved the use of an activity recorder, patterned after the description by Mitchell(8). Controls were run on the recorder for each cat before the operations. The instrument provided an easily standardizable method for determining whether a cat moved around the cage more after operation than before. Records were run on some animals throughout the day and night during alternate 15-minute intervals, by means of a timing device, and random recordings were made throughout the postoperative period. Visual observation combined with electrical recording provided an index of activity, and also presumptive evidence for sleep in these animals.

Five animals were fed a special diet prior to operation to determine the range of normal values for certain contemplated blood and urinary chemistry determinations. The diet consisted of 100 g of horse meat, 100 ml milk and 100 ml water per day. These animals were kept in metabolism cages which allowed accurate determination of urinary output. Daily rectal temperatures were also taken.

By means of cardiac puncture repeated samples of blood were obtained. Serum sodium and potassium were determined by flame photometry and chlorides by a Chloridometer. (Blood sugars were not determined in this experiment.) The values so obtained correlated well with those of other authors. Urinary chlorides were monitored using the "Fantus Test," and urinary reducing substances were determined by means of the Clinitest. The other 19 animals were fed a regular animal house diet. During the first 24 hours postoperatively 50 ml normal saline and 50 ml of a 5% glucose solution were administered subcutaneously.

The brains were removed after perfusion with 10% formol-saline. Alternate microscopic sections were stained by the Nissl and Weil technics for determination of the location and limits of the lesions.

Results. Microscopic evaluation of the 24 animals revealed that the lesions desired had been obtained in 11. This group displayed 2 forms of postoperative behavior. Seven of these 11 animals recovered uneventfully; however, it was noted and confirmed by the activity recorder that marked hyperactivity, *i.e.*, agitation and increased pacing associated with decreased sleeping time, occurred from the fourth to the seventh day. The remaining group of 4 recovered promptly but did not show this phase of hyperactivity.

Five of the 7 animals previously described, and 2 from the latter group of 4, began to decline physically after the seventh postoperative day, concomitant with failure to eat or drink, so that they had either died or were moribund at the time the brains were removed. Blood serum and urine chemistries failed to reveal any significant trends in the values obtained other than those which might be expected because of decreased oral intake, or the necessity for continued administration of subcutaneous fluids beyond the first postoperative day.

No abnormalities in temperature control, other than the transient drop found after pentobarbital anesthesia, was found in 5 animals so studied. Three of these were included in the group which declined to the moribund state.

The largest extent of a typical lesion is shown in Fig. 1. Rostral limits of the lesions in all 11 animals were at the level of the anterior commissure, as it crosses the midline,

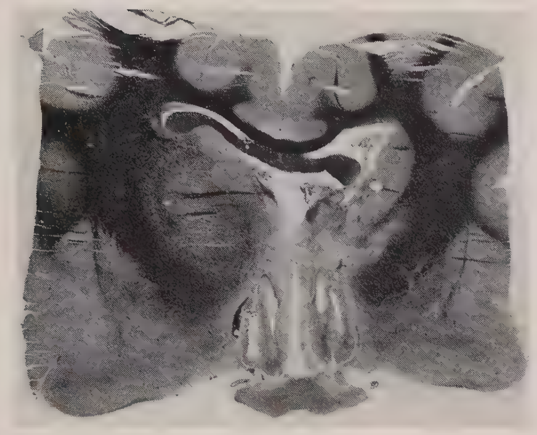

FIG. 1. Photomicrograph showing the greatest dimensions of satisfactory lesions in a typical animal. Weil stain.

and they extended caudally through the whole of the anterior hypothalamic area. Ventrally they reached the level of the optic chiasm and dorsally the roof of the third ventricle. However, when obvious microscopical differences were sought between the group of 7 cats that declined and the 4 that showed no change, no variations were readily apparent.

Discussion. Lesions located in presumably similar areas in the cat and rat appear to be without comparable effects on the sleep-waking cycle in the 2 species. The unexpected pattern of a sudden physical decline, beginning about the eighth postoperative day in a large percentage of cats, was interesting, but unexplained by such metabolic disturbance as might be indicated by the chemical methods herein used.

Without going into a minute analysis of fiber and nuclear damage and/or volumetric estimates of tissue damage, there appears to be no ready anatomical evidence for the difference in outcome of the 2 groups. Perhaps the eventual gathering of specifically meaningful knowledge of the area may help to account for these findings.

Summary. Eleven cats were subjected to lesions which were thought to correspond to Nauta's preoptic brain transections in rats. Seven of these exhibited a brief period of hyperactivity from the fourth through the seventh postoperative day, whereas 4 showed no change. Seven also declined physically, ceased drinking and eating, and died(2) or approached death before sacrifice. This decline could not be correlated directly with the periods of hyperactivity. There was no persistent alteration in sleep-wakefulness cycles. The only gross effect on serum electrolytes was concentration concomitant with dehydration. Potassium levels fell corresponding to a decrease in intake.

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Goldthioglucose-Induced Hypothalamic Lesion and ACTH Release.*
(26944)

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Goldthioglucose (GTG) when administered to mice in appropriate concentrations will produce localized destruction in the hypothalamus(1,2,3) and other areas of the

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TABLE I. Levels of Plasma Compound B in Animals of Various Groups.

	Controls	5 days post-GTG	Controls	18 days post-GTG
Resting levels	^{a} ♂ 8n* 18.1 ± 1.9†	^{c} ♂ 6n 56.8 ± 7.2	^{e} ♂ 10n 14.6 ± .7	^{g} ♂ 6n 14.4 ± 1.8
	^{b} ♀ 7n 24.5 ± 1.7	^{d} ♀ 6n 47.7 ±11.2	^{f} ♀ 8n 23.3 ± 1.6	^{h} ♀ 6n 25.5 ± 3.6
Stress: Ether	^{i} ♂ 4n 35.6 ± 2.1	^{k} ♂ 4n 46.2 ± 7.4	^{m} ♂ 4n 64.3 ± 7.8	^{p} ♂ 4n 68.3 ± 9.3
	^{j} ♀ 4n 87.6 ± 2.1	^{l} ♀ 4n 97.3 ± 7.4	^{o} ♀ 4n 89.3 ± 5.9	^{q} ♀ 4n 97.4 ± 1.9
Stress: Ether + fracture	^{r} ♂ 4n 35.6 ± 1.3	^{t} ♂ 4n 50.3 ± 3.9	^{v} ♂ 4n 69.5 ± 7.5	^{x} ♂ 4n 71.2 ± 4.3
	^{s} ♀ 4n 64.1 ± 4.4	^{u} ♀ 4n 78.6 ± 2.1	^{w} ♀ 4n 91.3 ± 1.5	^{y} ♀ 4n 112.5 ± 6.3

Statistical analysis: $^{a}/^{b}$, $P <.05$; $^{i}/^{j}$, $P <.01$; $^{r}/^{s}$, $P <.01$; $^{a}/^{c}$, $P <.01$; $^{k}/^{l}$, $P <.05$; $^{e}/^{m}$, $P <.01$; $^{f}/^{o}$, $P <.01$; $^{i}/^{k}$, N.S.; $^{j}/^{l}$, N.S.

* This figure followed by n indicates No. of animals in the group.

† Concentrations of plasma-free corticosteroids expressed in μg Compound B/100 ml plasma ± S. E. (The superscript letter in front of the set of figures of each group permits rapid identification of the group for expressing the statistical analysis: $^{a}/^{b}$ = figures of group ^{a} compared with those of group ^{b}, etc.)

brain(4). Animals with such lesions show an increase in food intake(5,6) and subsequently obesity(7,8,9,10). In animals of the CBA strain, extent of the lesion in the diencephalon as well as the course of the ensuing obesity, are predictable for a given dose of GTG, in contradistinction to variability of results observed in other strains after injection of the drug(11). The hypothalamic lesion produced by GTG destroys areas of the ventral hypothalamus which in many reports (review in 12) have been implicated in control of the secretion of adrenocorticotropin (ACTH). The purpose of these experiments was to investigate whether the hypothalamic lesion regularly produced in CBA mice by GTG would inhibit the release of ACTH that ensues upon exposure to stress. In view of the simplicity of producing such a lesion, as compared to methods that use stereotaxic technics, our primary interest was in establishing whether GTG injection could provide an easy assay animal for studies on corticotropin releasing factor (CRF). Effects of the GTG-induced hypothalamic lesions on the resting pituitary-adrenal function, *i.e.*, in absence of stress, were also studied.

Methods and materials. One hundred twenty-one male and female mice of the CBA inbred strain§ were used. Animals were housed, 4-6 per cage (6″ × 12″ × 6″) and fed Purina laboratory chow *ad libitum*. Levels of pituitary-adrenal function were assessed by measurement of plasma free corticosterone (Cpd. B) levels(13,14). This was done on a 0.2 ml plasma sample from individual animals(13). Resting levels were obtained in animals sacrificed by instantaneous decapitation. All blood samples were obtained by decapitation, collecting the blood in heparinized tubes. The experimental protocol shown in Table I was designed to study in males and females the effects of goldthioglucose-induced hypothalamic lesions on the functional status of the pituitary-adrenal system during "resting" conditions and in response to acute stress as compared to untreated, control animals. It was decided to study the animals at 5 days and 18 days after injection of GTG. Previous experiments have shown(2) that at 5 days post GTG, the hypothalamic lesion is fully developed (Fig. 1). With the schedule at 18 days post GTG it was proposed to study pituitary-adrenal function in these animals when in the "active phase" of the weight gain leading to obesity (6). Two types of stress were used: ether anesthesia alone, or combined with fracture of one femur. In both cases, blood samples for plasma Cpd. B measurements were obtained exactly 15 minutes after application of the stress. All animals were sacrificed at the same time of day (between 1:00 p. m. and 3:00 p. m.), to minimize the 24 hour variation in plasma corticoid levels which has been reported in mice(15) and rats(16).

GTG‡ was administered as a single, i. p. injection of 0.4 mg/g body weight in animals

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§Obtained from Kirschbaum Memorial Research Laboratory, Dept. of Anatomy, Baylor Univ. College of Medicine.

‡ Courtesy of Schering Corp., Bloomfield, N. J.

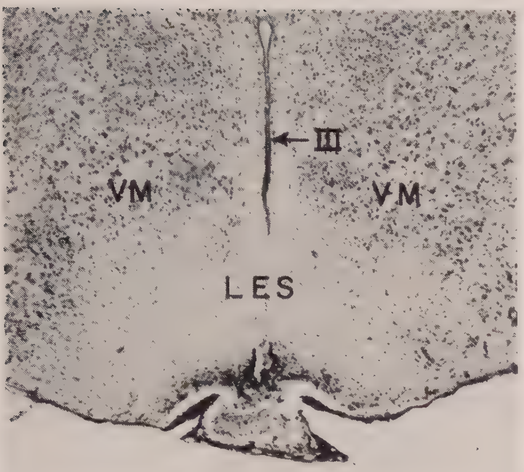

FIG. 1. Goldthioglucose-induced lesion in ventral hypothalamus of CBA mouse 5 days post-injection (V.M.—ventromedial nucleus; LES—lesion; III—third ventricle). Dose—0.4 mg/g body weight. Stain—thionine. x 45.

of 90-120 days of age(2). The various groups of animals shown in Table I were studied at either 5 or 18 days after injection of GTG. Brains of all GTG-treated animals killed at 5 days post-injection were fixed in 10% formalin. Midline sagittal reconstructions (frozen sections, 50 μ, thionin) were made for 8 brains to determine size and location of the hypothalamic lesion, and the stained sections of the remaining brains were examined histologically.

Results. Results are summarized in Table I. The normal control females had higher levels of plasma Cpd. B than the males under "resting" and stress conditions as previously reported by others(15,17). Five days after injection of GTG, at a time when the hypothalamic lesion was fully developed, levels of circulating plasma Cpd. B in both males and females were higher than in control animals, when measured in resting conditions, *i.e.*, in absence of stress. Exposure to stress led to no increase of these high levels in the male whereas in the female further increases were seen. Eighteen days after injection of GTG, resting levels of plasma Cpd. B in males or females had returned to normal levels as compared to normal control animals; exposure to stress was followed by significant elevations of plasma Cpd. B levels, similar to those observed in the normal control animals subjected to stress.

Fig. 1 shows a typical hypothalamic lesion as seen in an animal sacrificed 5 days after administration of GTG. This lesion was a constant finding in *all* animals injected with GTG and corresponds to what has been reported previously(2,4). A midsagittal diagram of the hypothalamus shows the anterior and posterior extent of the lesion (Fig. 2). The only minor variations observed were in the size of the lesions. All animals 18 days after GTG showed considerable increase in body weight when compared to their controls (males, +20.6%; females, +29.6%).

Discussion and conclusions. The increase in plasma corticosterone resting levels seen in the first few days after injection of GTG may be due to one or several mechanisms: alteration of the conjugation capacity of the liver(18) due to the drug itself or accompanying the diminished food intake that immediately follows GTG injection(6), thus

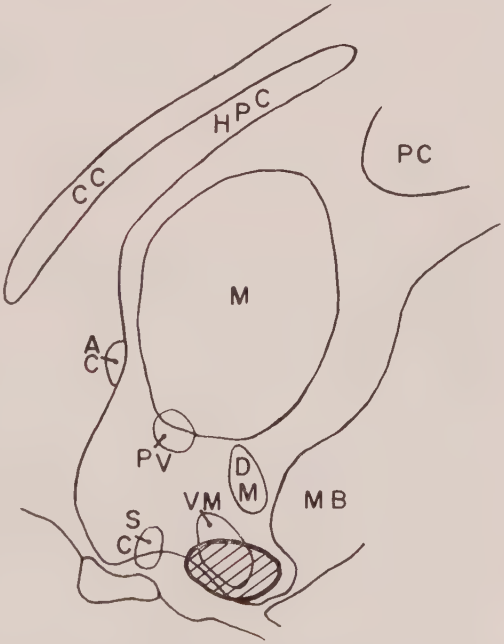

FIG. 2. Midsagittal diagram of the hypothalamus localizing a goldthioglucose-induced lesion in a CBA mouse at 5 days post-injection. Abbreviations used: CC, corpus callosum; DM, dorsomedial nucleus; M, massa intermedia; MB, mammillary body; PV, paraventricular nucleus; VM, ventromedial nucleus; SC, suprachiasmatic nucleus.

leading to increased plasma concentrations of *free* Cpd. B; or actual stimulation of the hypothalamic centers related to ACTH release by development of the hypothalamic lesion.

These results show, at any rate, that the hypothalamic lesion produced in CBA mice by the dose of GTG used in this study does not inhibit release of ACTH normally produced by exposure to stress. Animals with this type of hypothalamic lesion, therefore, cannot be used for an assay of CRF.

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Extension of Fertilizability in the Sand Dollar Egg with Vitamin B$_{12}$.*

(26945)

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Unfertilized eggs of the sand dollar, *Dendraster excentricus,* lose the capacity to be fertilized within 48 hours when allowed to stand uncrowded in fingerbowls at 18°C. Addition of cobaltous chloride to the sea water will increase the fertile life of the eggs 4-8 days. When sulfhydril compounds are added to the proper concentrations of cobalt in sea water the fertile life may be extended to as much as 25 days(1,2). In view of these findings it seemed desirable to determine the effect of cyanocobalamin (Vit. B$_{12}$) on fertilizability.

Methods. Mature sand dollars were taken from Monterey Bay and maintained in aquaria at the Hopkins Marine Station, Pacific Grove, Calif. Ova were removed from ripe females and washed several times before being placed in various concentrations of the vitamin. Since the crystalline form of the vitamin was not available at the time of experiments a sterile aqueous solution (Squibb's Rubramin–containing 1,000 μg Vit. B$_{12}$ per cc in isotonic NaCl with 1.5% benzyl alcohol as a preservative) was diluted with sea water. As simple dilution with sea water also diluted the benzyl alcohol it was necessary, in some cases, to add benzyl alcohol to the diluent to maintain a constant concentration of the preservative while varying the concentration of the vitamin. All cultures were kept in covered fingerbowls, out of direct sunlight and at 18 ± 1°C. Fertility was determined at 24-hour intervals by addition of fresh

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* The author is grateful to the Hopkins Marine Station, Pacific Grove, Calif., for use of its facilities and to the Graduate School, Northwestern University for material support during this work.

TABLE I. Extension of Fertility in Eggs of *Dendraster excentricus* with Vitamin B_{12} and Benzyl Alcohol.

Solution used	24 hr	48 hr	72 hr	96 hr	120 hr
	%				
Sea water (control)	80	—	—	—	—
.25% benzyl alcohol*	20	—	—	—	—
.15% " "	80	50	10	—	—
100 µg vit. B_{12}/cc in .15% benzyl alcohol	90	85	40	35	—
50 µg *idem*	90	75	40	30	30
25 µg "	90	75	40	3	—
12.5 µg "	80	60	30	20	10
6.25 µg "	90	60	40	30	20

* Benzyl alcohol solutions made up with sea water.

sperm suspension to washed samples of approximately 100 eggs. The criterion of fertilization was nuclear division and the number fertilized usually was recorded to the nearest 5.0%.

Results. Vitamin B_{12} clearly extended fertilizability in sand dollar eggs (Table I). None of the control eggs was fertilizable at 48 hours. In 5 different concentrations of B_{12} (100 µg-6.25 µg per cc) in 0.15% benzyl alcohol fertility was extended beyond that of benzyl alcohol alone. It was evident that loss of fertility in these eggs was delayed by the vitamin. Since benzyl alcohol alone was found to preserve fertility it seemed advisable to determine its effects at various strengths. It was found that 0.25% benzyl alcohol (Table I) was toxic enough to decrease while 0.15% (Table I) and 0.075% (Table II) extended (and with 0.075% enhanced) fertilizability in these eggs. With lower concentrations (0.0375%, 0.009375% – Table II) the percentage fertilizable at 24 hours was decreased but there was some extension of the fertile period.

In another set of experiments the concentration of B_{12} was kept constant while the strength of the benzyl alcohol was varied. As noted above (Table I) 6.25 µg of the vitamin per cc in 0.15% benzyl alcohol prolonged the fertilizable life of 20% of the eggs for over 120 hours. When this same quantity of B_{12} was used with benzyl alcohol of lower concentrations (0.075%–0.009375%) the preserving effect was not significantly better than benzyl alcohol alone (Table II). It seems clear from these data that low concentrations of B_{12} in sea water require benzyl alcohol (or something similar) in proper concentration to prolong fertilizability in these eggs.

Discussion. The fertile life of various marine eggs has been extended by means other than temperature. Low calcium sea water prolongs the fertile life of *Arbacia* eggs(3) while the fertile life of *Urechis* eggs has been extended with ethyl alcohol and dextrose (4). Various amino acids and proteins have been used to improve fertilization (but not to preserve fertility) in echinoderm eggs(5). It has been suggested that fertility may be preserved by preventing internal clotting, by

TABLE II. Extension of Fertility in Eggs of *Dendraster excentricus* with Vitamin B_{12} and Benzyl Alcohol.

Solution used	24 hr	48 hr	72 hr	96 hr	120 hr
	%				
Sea water (control)	80	—	—	—	—
.075% benzyl alcohol*	95	95	1	—	—
.0375% " "	70	40	—	—	—
.009375% " "	50	5	—	—	—
6.25 µg vit. B_{12}/cc in .075% benzyl alcohol	95	90	5	—	—
Idem .0375% " "	80	50	—	—	—
" .009375% " "	70	—	—	—	—

* Benzyl alcohol solutions made up with sea water.

supplying nutritional substances, and/or by decreasing permeability.

Loeb(6) suggested that the starfish egg normally becomes unfertilizable after a damaging amount of aerobic oxidation takes place, and believed that the slowing down of this action (through use of potassium cyanide and low oxygen) was responsible for prolonging fertilizability in these eggs. The writer(2) has suggested that cobalt prevents deterioration of fertilizability by uniting with (and preventing oxidation of) thiol groups at the egg surface. The action of Vit. B_{12} in preserving fertility may well be through protection of the egg surface from destructive oxidations. It seems not unlikely that B_{12} combines with surface proteins in much the same way that Co^{60} Vit. B_{12} combines with serum proteins (alpha and beta globulins) and the protein of cerebro-spinal fluid(7). The preserving action of benzyl alcohol alone may be in the retardation of the processes (oxidations, increased permeability, etc.) which normally lead to the decay of fertility.

Summary. Vitamin B_{12} (100-6.25 μg/cc) in the presence of 0.15% benzyl alcohol extends fertilizability in the eggs of *Dendraster excentricus*. Benzyl alcohol alone also has some preserving action. It is suggested that both substances prolong fertility by inhibiting destructive processes that occur with aging of the eggs.

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Effects of Clotting and Clot Lysis on Platelet Thromboplastic Activity.* (26946)

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During coagulation of shed blood the majority of platelets undergo clumping, "viscous metamorphosis",(1,2) and, eventually, disappear from the liquid phase. Presumably these platelets are either trapped in the fibrin meshwork of the clot or disintegrated. The few platelets which remain in the liquid phase have been called "serum platelets" and their properties described by Alexander(3). Platelets contain a thromboplastic factor (platelet factor 3) which is essential to normal blood coagulation. These studies were undertaken to determine what happens to this factor during and after clotting. To avoid interference from red cells, platelet-rich plasma was used. To free platelets from enmeshing fibrin network, clots were either chopped and ground or lysed.

Materials and methods. Platelet-rich plasma. One unit of ACD blood, drawn in a plastic bag, was centrifuged at 400 g for 25 min.; the plasma was transferred to plastic tubes and recentrifuged at 300 g a number of times until essentially free from red cells. *Streptokinase* (SK), Varidase, was kindly supplied by Dr. Ruegsegger of Lederle Laboratories. *Platelet-thromboplastic factor* was estimated by 2 methods: (a) a standard TGT test(4) in which equal volumes of normal $BaSO_4$ adsorbed plasma (dil. 1-5), normal serum (dil. 1-10), platelets and 0.025 M $CaCl_2$ were mixed and tested for thromboplastic activity every minute by adding 0.1 ml to 0.2 ml recalcified normal substrate plasma. Minimum clotting time of the latter is reported.

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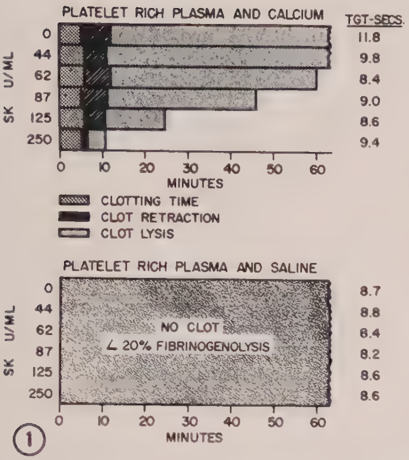

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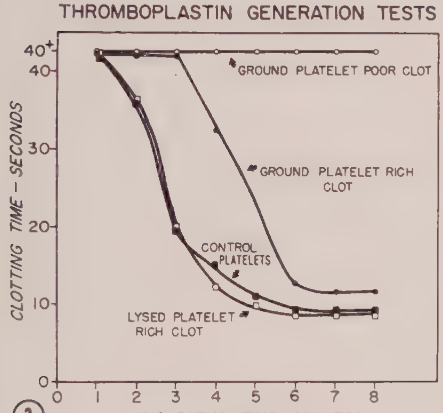

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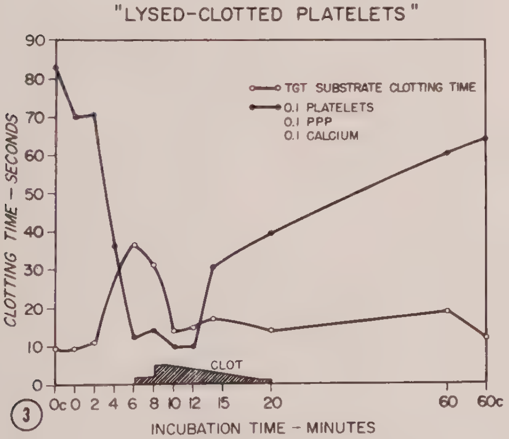

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FIG. 1. TGT substrate clotting times from mixtures containing platelet sediments from platelet-rich plasma with varying amounts of streptokinase, with and without calcium.

FIG. 2. Effects of control platelets, clot-lysed platelets, ground platelet-rich clot and ground platelet-poor clot on thromboplastin generation using standard $BaSO_4$ plasma and serum.

FIG. 3. Effects of incubation before decalcification of mother tube on platelet-thromboplastic activity.

(b) The second method recorded the clotting time of a mixture of 0.1 ml platelet-poor plasma (normal plasma centrifuged 60 min. at 20,000 g) plus 0.1 ml platelets or saline plus 0.1 ml 0.02 M $CaCl_2$.

Results. Effects of streptokinase concentration and clotting on platelet-thromboplastic factor. Fig. 1 shows the results of an experiment in which platelet-rich plasma was distributed in 4 ml amounts in 12 new glass 13 × 100 mm tubes; 0.1 ml appropriately diluted streptokinase was added to each tube to give the final concentrations shown in the figure, and 0.1 ml 0.8 M $CaCl_2$ was added to the first 6 tubes and 0.1 ml saline to the last 6. Clotting time, clot retraction time and clot lysis time of the first 6 tubes were recorded. After 60 min. all were centrifuged at 20,000 g for 15 min., the sediments washed twice and suspended in 0.4 ml saline. Sediments from tubes 1 and 2 were chopped and ground. All sediments were diluted 1-10 for TGT, the results of which are shown in the last column.

Streptokinase alone had no effect upon the activity of platelets in the TGT test and caused little fibrinogenolysis of the mother plasma. Clotting and subsequent clot-lysis also had little or no effect on TGT time, although the sediments from tubes 1 and 2 were very difficult to suspend smoothly.

Fig. 2 illustrates another experiment in which the TGT curves of control platelets, sediment from a lysed platelet-rich clot, sediment from a ground platelet-rich clot and sediment from a ground platelet-poor clot are compared. The "clot-lysed" platelets appear entirely similar to the control unclotted platelets. The ground washed platelet-rich clot contained significant amounts of thromboplastic factor, although again it proved difficult to prepare a fine suspension. The ground platelet-poor clot showed no platelet-thromboplastic activity.

Sediments were also tested for complete prothrombinase (or plasma thromboplastin) activity by testing in TGT systems deficient in $BaSO_4$ plasma, in AHF (using Hemophilic $BaSO_4$ plasma), in serum and in PTC (using PTC deficient serum). All tests showed no generation of plasma-thromboplastin or prothrombinase unless both normal $BaSO_4$ plasma and normal serum were present. Sediments were also tested for presence of thrombin by adding fibrinogen solution. No clots were formed in 6 hours. Recalcification times of platelet-poor plasma to which 0.1 ml full-strength platelets or "clot-lysed" platelets were added were similar.

In another experiment (Fig. 3), an attempt was made to stop coagulation at various intervals after recalcification by mixing 0.1 ml 1 M sodium citrate into the coagulation mixtures containing 4 ml platelet-rich plasma, 0.1 ml streptokinase (1000 units) and 0.1 ml 0.8 M $CaCl_2$. The tubes were chilled and centrifuged immediately after addition of citrate. The sediments were washed twice, suspended in 0.4 ml saline and tested full strength in the recalcification test and at a 1-10 dilution in the TGT. Tubes 0_c and 60_c were not recalcified. As shown in Fig. 3 there was a rapid decrease in the platelet-poor plasma recalcification time employing sediments from tubes which had been recalcified from 2 to 12 min. earlier. Thereafter activity decreased, nearly reaching control levels 60 min. after the platelet-rich plasma had been recalcified. This apparent increase in "thromboplastic activity" of the platelets (or sediment) occurred simultaneously with beginning clot formation in the mother tube. It was due, at least in part, to the presence of thrombin, as, even after two washings, these platelets clotted (in 60 secs. or longer) fibrinogen or plasma *without* the addition of calcium. At the same time what appeared to be a paradoxical lengthening of the TGT substrate clotting time was often observed. In one experiment the control platelets produced a 9.8 sec. TGT clotting time and the 6 min. platelets a 36.8 sec. time.

In another experiment, shown in Table I, platelets from the unclotted SK control tube and a 7 min. clot-lysed tube were diluted to various strengths and then used in the standard TGT test. The substrate clotting times after 1 min. and 6 min. incubation of the TGT mixtures are shown. The 100% platelet dilution was that in which the platelets were suspended in a volume of saline equal to that of the platelet-rich plasma from which they were obtained. As expected, when control platelets were diluted the substrate clotting times after both 1 min. and 6 min. of incubation became longer. Rather surprisingly, the platelets from the 7 min. clot-lysed tube acted in a reverse fashion, the TGT substrate clotting times becoming progressively shorter with dilution down to 25%. This experiment has been repeated a number of times with similar results.

TABLE I. TGT Substrate Clotting Times 1 and 6 Min. after Incubation of Dilutions of Control and 7 Min. Clot-Lysed Platelets in Standard TGT Mixtures.

	TGT substrate clotting time (sec.)			
	Control		7 min. clot-lysed	
	TGT incubation time (min.)			
Dilution, %	1	6	1	6
100	18.2	7.6	18.2	16.2
75	27.0	8.6	11.8	9.2
50	30.2	9.6	10.8	7.8
25	40+	10.8	10.0	8.0
10	40+	12.8	11.8	11.2
5	40+	18.6	19.6	14.6

Platelets from the control tube (Table I) did not contain thrombin and were unable to clot plasma without additional calcium. Those from the 7 min. tube clotted plasma or fibrinogen in approximately 60 sec. in dilution similar to that in the TGT mixture. In an attempt to demonstrate a possible TGT inhibiting effect of thrombin, platelets were incubated with various dilutions of bovine thrombin (250 to 0.1 unit), washed twice, and tested in a standard TGT test. Although the 1 min. substrate clotting times were shorter than that with the untreated platelets, the 6 min. times were identical.

In another experiment, Table II, platelets from the unclotted SK control tube and a 7 min. clot-lysed tube were heated for 10 min. in a boiling water bath or frozen-thawed before testing. Heating and freezing both shortened the minimal substrate clotting time obtained after 7 min. incubation of the TGT mixture. In addition heating of the clot-lysed platelets resulted in a longer 1 min.

TABLE II. TGT Substrate Clotting Times (Sec.) 1 and 7 Min. after Incubation of Untreated, Heated and Frozen Control and 7 Min. Clot-Lysed Platelets.

	Untreated		100°C, 10 min.		Frozen-thawed	
	TGT incubation time (min.)					
Platelets	1	7	1	7	1	7
Control	40+	9.8	40+	10.0	40+	9.8
7 min. clot-lysed	23.0	13.0	40+	10.0	21.0	10.8

TGT substrate clotting time, perhaps due to destruction of contaminating thrombin.

It was also noted that the platelets from the early phases of clotting did not contain full plasma-thromboplastin activity as both $BaSO_4$ treated plasma and serum were necessary for their full activity in the TGT test.

Comment. These experiments show that "platelets", even after clotting, retain their full platelet-thromboplastic or factor 3 activity. After clotting and clot lysis the platelets have a shaggy appearance but are still recognizable with the phase microscope. It is possible that the thromboplastic activity is present in small granules outside of the platelet shell which sediment at the same speed, although such granules could not be distinguished microscopically. The chopped ground clot sediment had no ability to retract clots. The sediment from the lysed clotted samples, even after many washings, caused lysis of plasma clots. Additional experiments showed that human platelets contain a streptokinase cofactor (? proactivator) and when added with streptokinase to bovine profibrinolysin produce an active fibrinolysin.

Platelets obtained from recalcified platelet-rich plasma in the early stages of clotting retain traces of thrombin. They are able to clot platelet-poor plasma without added calcium and, in the presence of calcium, produce very rapid clotting. The same platelets when used in a TGT test promote lessened evolution of plasma thromboplastin unless they are first diluted or heated. This apparent inhibition is reminiscent of the poor evolution of plasma thromboplastin observed by Spaet(4) when very concentrated platelets were used. The anticoagulant activity of Spaet's platelet lipid was stable at 100°C., while that found in defatted platelets was destroyed by heat. The apparent anticoagulant activity of platelets separated from lysed samples soon after recalcification is destroyed by 100°C, leaving only the coagulant activity.

Summary. Sediments obtained from platelet-rich plasma allowed to clot for 1 hour contain full platelet-thromboplastic or factor 3 activity. Sediments obtained from recalcified platelet-rich plasma in the early phase of clotting contain thrombin and appear to be inhibitory to normal thromboplastin generation.

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The Cytostat, An Apparatus for Automatically Controlled Continuous Propagation of Suspended Cells.* (26947)

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Designs for continuous cultivation of cells in suspension have been introduced particularly for the study of bacteria, algae and

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fungi. Myers and Clark(1), Bryson(2) and Anderson(3) described the *turbidostat* principle for continuous cultivation of bacteria. Here, as turbidity increases in proportion to cell density, fresh medium is supplied while an equivalent quantity of cell suspension is removed.

A different principle of cell cultivation is used in the *chemostat,* introduced by Monod (4) and at about the same time by Novick and Szilard(5). Here medium is supplied continuously to the cell culture and an equivalent quantity of cell suspension is removed simultaneously. Rate of flow of the medium is so adjusted as to make the culture self-limiting and to achieve a *steady-state.* A prerequisite for this control is that the medium contains a limit concentration of some essential metabolite.

Attempts have also been made to propagate suspended mammalian cells of several types *in vitro.* Owens, Gey and Gey(6) described how they cultivated lymphoblastic mouse tumors in rotating tubes. Earle *et al.* (7) reported success in growing L-fibroblasts in suspension in flasks mounted on a Brunswick agitator. Cherry and Hull(8,9) and McLimans *et al.*(10) described the stirrer or spinner culture, where cells are agitated by a rotating magnetic stirring bar suspended by means of a nichrome wire. Interesting results were obtained with a large number of established mammalian cell lines. In these cases, however, the entire culture medium or a large part thereof was changed at various intervals (batch principle). A steady-state of growth was never achieved or attempted.

It is uncertain whether the turbidostat has been successfully adopted for controlled growth of mammalian cells. The chemostat, however, has been used for propagating Earle's L-fibroblasts and a simplified chemostat for growth of mammalian cells was recently introduced by Cohen and Eagle(11), who described the characteristics of continuous growth of the cell line HeLa-S3, KB and liver. A similar principle was utilized in the so called cytogenerator of Graff and McCarty (12).

In the rapidly developing field of cell culture *in vitro* two trends are clearly seen: (1) interest in meeting future undefined needs to grow mass cultures of mammalian and other cells; (2) the necessity for obtaining basic information about cell reactions to environment(13) particularly under steady-state conditions.

The turbidostat is useful for a number of applications in bacteriology. However, in a medium with distinct optical properties, determination of concentration of cells with light scattering surface will have to depend on complex relationships between scattered, transmitted and absorbed light. For regulation or direct readings of cell concentration the turbidostat principle is therefore not ideal.

The chemostat has the advantage of simplicity but operates under conditions of some growth-limiting metabolite which may be comparable to cell starvation. It is not known to what extent the limiting factor changes the metabolism of the cell and how the cell, grown under such conditions, compares with a cell propagated in an environment with no other limitations than those inherent with the cell.

To study this and other related problems the *cytostat* (I.R.L.2) was developed. It permits use under conditions when either the cell concentration or a limiting essential metabolite concentration is used as the controlling factor. Intermittent readings of cell density can be made and growth comparisons of a number of simultaneous cultures are possible. It has also proved itself useful for pilot experiments for purposes of mass propagation in steady-state. These functions are carried out automatically. Fig. 1 shows the cytostat in which the turbidometric principle was replaced by a nephelometric device. As this construction has proved a useful aid to a number of *in vitro* studies of cell populations, a description is given.

The Cytostat comprises a heat-insulated Plexiglass housing which can accommodate up to 8 silicone-treated culture vessels containing 50-1500 ml. Nutrient medium is fed by tube to each culture vessel and the volume of each individual culture is regulated by an adjustable drainage tube which maintains constant level by withdrawing cell suspension

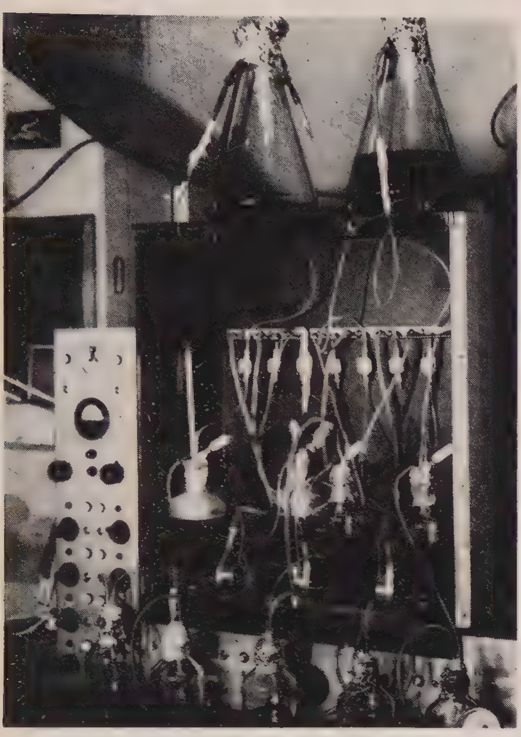

FIG. 1

as soon as the supply of fresh medium raises the level above the drainage tube. Rotating magnets provide cell suspension agitation, a double thermostat and simmerstat maintain constant temperature, a fan circulates air in the Plexiglass housing and individual gas circulators pump suitable gas through the culture vessels.

When used as a chemostat, the medium is pumped continuously into the culture vessels. When, however, cell concentration is used as the controlling factor, *a nephelometric unit,* (Fig. 2) is used. This consists of *a circular light source and a photocell,* (Fig. 3) and is placed on *a cylindrical projection on the wall of the culture vessel* (Fig. 4) and a sensitrol relay is set to operate at a given cell concentration. A rheostat controls the intensity of the light falling on the cells and the photocell-rheostat system can be used to read cell concentration at chosen intervals after suitable calibration.

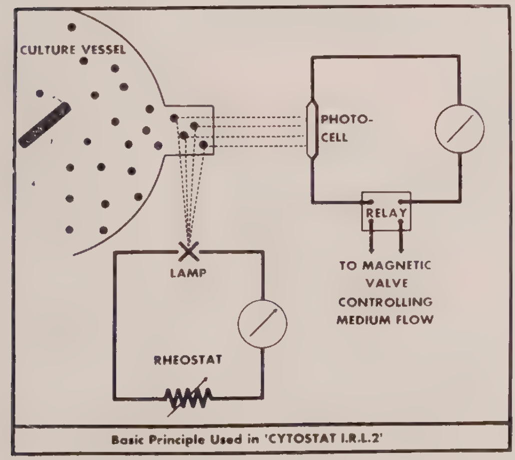
CULTURE VESSEL
PHOTO-CELL
RELAY
LAMP
TO MAGNETIC VALVE CONTROLLING MEDIUM FLOW
RHEOSTAT
Basic Principle Used in 'CYTOSTAT I.R.L.2'

FIG. 2

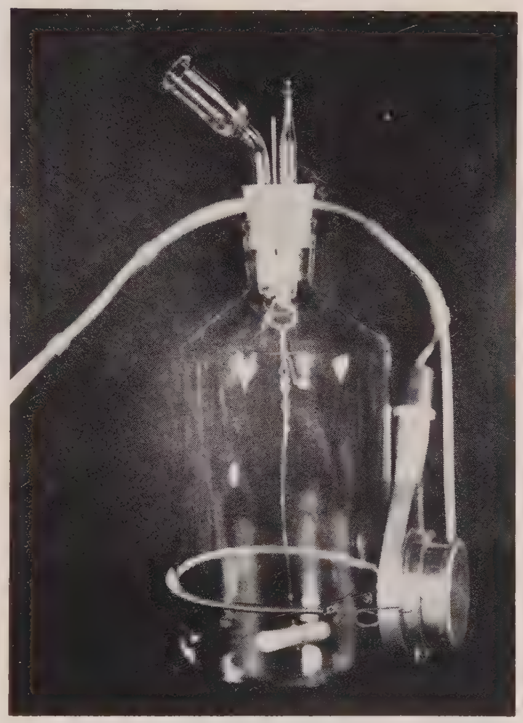

FIG. 3

A programmed, automatic reader (Fig. 5 and 6) records hourly the cell concentration of each culture vessel. Whenever cell density exceeds a set limit, a silicone-treated magnetic valve opens to allow addition of fresh medium to the culture until it is diluted to the set concentration. An equivalent quantity of cell suspension is simultaneously removed by the partial vacuum created in the drainage tubes by the gas circulators.

The critical detail is the photocell light-source sensitrol-relay combination. Dowex

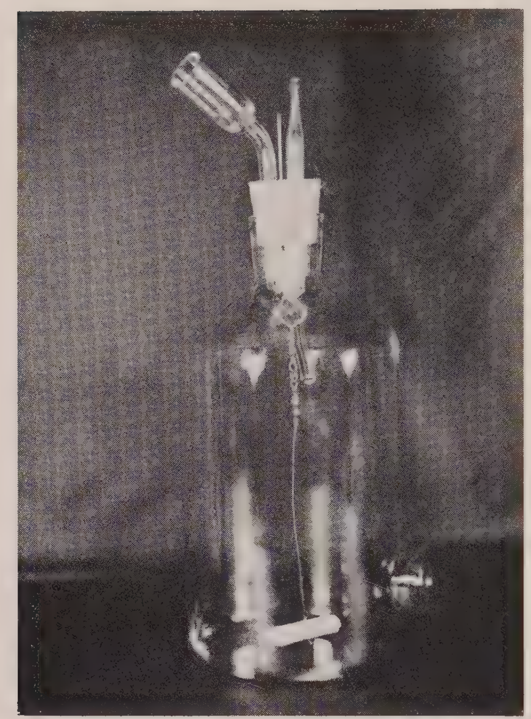

FIG. 4

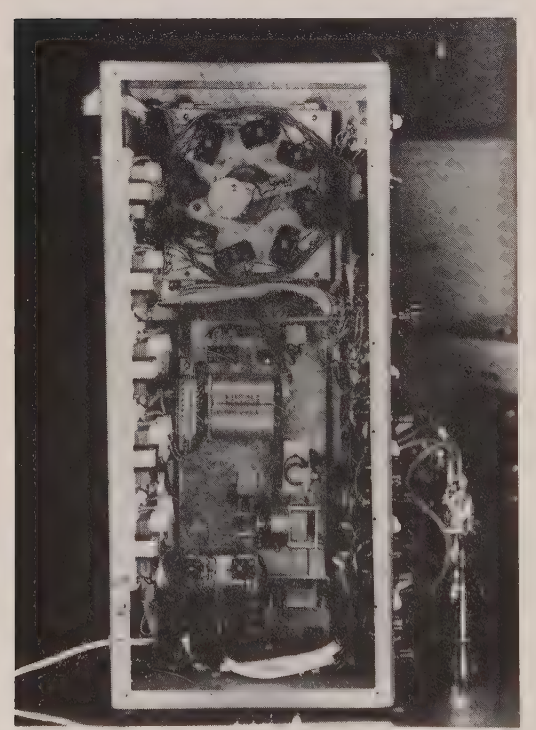

FIG. 5

particles, red blood cells and HeLa cells were used to test it. Fig. 7 shows the intensity of the incident light (lamp voltage) as a function of the cell density of the suspension at a constant scattered light. Readings were made at 2 different intensity levels. Even though Dowex particles reflect light comparatively weakly, it is apparent that straight-line graphs are obtained at both levels and that they are almost parallel if the logarithmic scale is taken into consideration. It is also apparent that light intensity, meas-

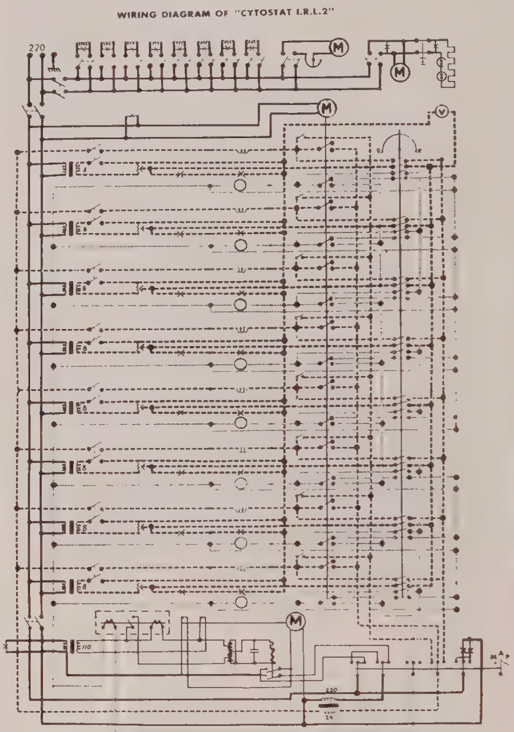

FIG. 6

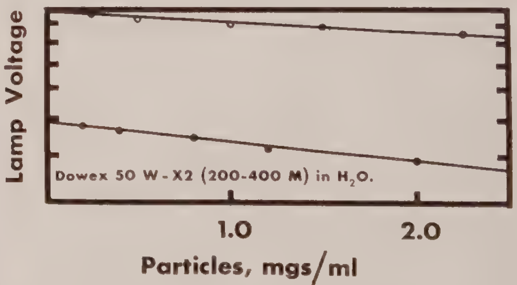

FIG. 7. Two levels of incident light versus cell concentration at constant scattered light.

ured here as a function of lamp voltage, plays no deciding role.

Fig. 8 shows how much more strongly human red blood cells scatter incident light than do Dowex particles. The intensity level of the scattered light may be kept constant either by using high cell concentration or high lamp voltage.

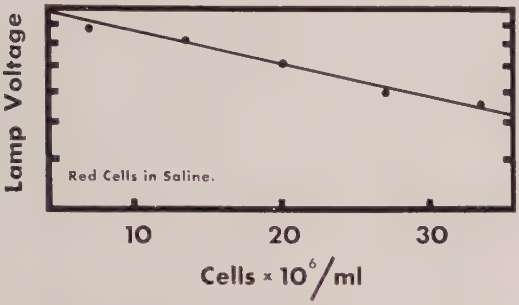

FIG. 8. Incident light versus cell concentration at constant scattered light.

The relation between incident light and cell concentration at a constant level of scattered light is not influenced to any special degree by a serum content of up to 40% of the medium or addition of phenol red to the medium (Fig. 9). But because different photocells react differently, it is important that the type chosen be especially sensitive to light in the yellow and red spectrum and not particularly dependent on ultra-violet light, which is absorbed by proteins.

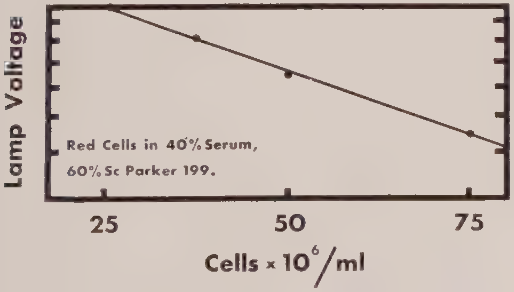

FIG. 9. Incident light versus cell concentration at constant scattered light.

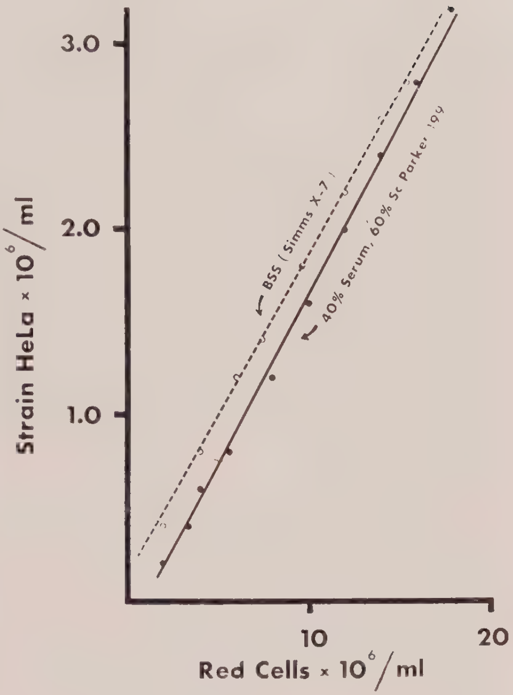

FIG. 10. Iso-nephelometric relations of human red cells and HeLa.

A comparison of HeLa cell and human red blood cell suspensions shows that either type of cell can be used for nephelometric cell concentration determinations, whether in balanced saline or in serum-containing media. Fig. 10 shows the practically parallel straight-line relationship between HeLa cells and red blood cells, measured nephelometrically, and

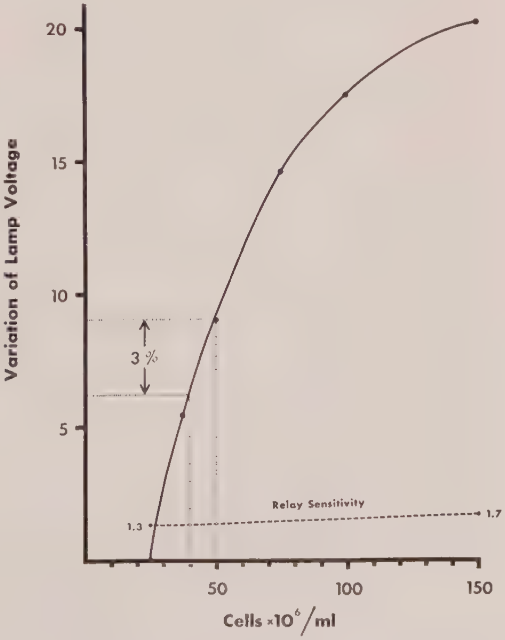

FIG. 11. Percentage variation of incident light versus cell concentration at constant photocell output.

that a HeLa cell scatters as much light as 5 or 6 red blood cells. The latter can therefore be used for checking and adjusting the optical system.

The sensitivity of the control system may be judged from Fig. 11 which shows that a 3% alteration of the incident light is necessary to maintain a constant level of scattered light if the cell density increases from 40 to 50 million/ml. Since the sensitivity of the sensitrol relay is better than 1.5%, it is clear that such an increase of cell density cannot occur without the relay operating and supplying fresh medium. Within this concentration range, the system has a sensitivity to change of about ± 5%.

Summary. The cytostat and its operation have been described. It would seem to be a useful aid to further *in vitro* studies of cell populations.

The authors owe thanks to Mr. Tage Axelsson and Mr. Artur Andersson for skillful and devoted contributions.

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Recovery of Reoviruses from Wild and Laboratory Mice. (26948)

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Representatives of the ECHO 10 family, recently designated as the reovirus group(1), have been isolated from man, chimpanzees, monkeys, and cattle, and serologic evidence of infection has been detected in guinea pigs and other mammalian species(1,2). This report describes the isolation of naturally occurring viruses serologically related to reovirus types 2 and 3 from wild mice and laboratory mice, respectively.

Materials and methods. Tissue cultures. Primary trypsinized mouse embryo (ME) and rhesus monkey kidney (MK) tissue cultures were obtained from Microbiological Associates, Inc., Bethesda, Md. Maintenance fluid for ME cultures was 5% horse serum in Eagle's Basal Medium, which was replaced twice weekly. MK cultures were maintained in Mixture 199, without intercurrent fluid change; in later tests, 1% calf serum without gamma globulin (Newborn Agamma calf serum, Hyland Labs., Los Angeles, Calif.) was added. All media contained penicillin (250 u/ml) and streptomycin (250 μg/ml).

Mice. For experimental inoculations, weanling and newborn Swiss mice, strains "General Purpose" and "NIH", were obtained from the Animal Production Section, Nat. Inst. of Health. Mice tested for virus were sacrificed within 24 hours after receipt in the laboratory. Wild mice were live-trapped.

Viruses. As reference viruses, the following reovirus strains were used: type 1, SV

12; type 2, D5; and type 3, Abney. These strains were kindly supplied by Dr. Leon Rosen, who has described their origin(2,3). Standard virus pools for hemagglutination and neutralization tests consisted of supernatant fluids plus cells from infected MK tissue cultures. Pools were stored at 4° or −60°C.

Antiserum. Immune serum was prepared in groups of 20 to 50 weanling mice by giving a single intraperitoneal inoculation of 0.1 or 0.2 ml undiluted MK tissue culture virus, and bleeding 3 weeks later. In some cases a second injection was given at 3 weeks, with bleeding 7 to 10 days later. Individual mouse sera were obtained by orbital puncture or by decapitation, and were prediluted as described previously(4).

Virus isolation. Virus was obtained from suspensions of solid viscera (liver, spleen, kidneys, pancreas, gonads, salivary glands, and brain) or intestines of normal mice, and from transplant-induced tumors. In the course of surveys for mouse polyoma virus, these materials were subjected to one or more of the following virus detection procedures:

1) Tissue culture. 0.05 to 0.1 ml of tissue suspension clarified at 2000 rpm for 15 to 20 minutes was inoculated into 2 to 5 ME or MK tissue cultures, or both. ME cultures were observed for 3 to 6 weeks and passages were made to ME and MK tissue cultures whenever tissue destruction or cellular alteration was noted. With MK tissue culture, blind passages were made routinely at 19 to 21 days, or earlier if definite tissue destruction was noted; cytopathic changes identifiable as those caused by reoviruses were usually not seen in the primary inoculation tubes.

2) Mouse antibody production (MAP) test: Three to 5 weanling mice were inoculated intraperitoneally with 0.1 to 0.2 ml of test material; the mice were bled individually in 28 to 35 days and the sera tested for hemagglutination-inhibiting antibody. Appropriate control mice were included in the test.

3) Newborn mice: One or 2 litters of newborn mice were inoculated with 0.04 ml intraperitoneally; one-half of each litter was sacrificed at 7 days and a suspension of pooled viscera inoculated into a second group of newborn mice, into weanling mice for MAP testing, and into tissue culture. The remaining mice were observed for illness for two weeks.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests. HA and HI tests were performed in tubes, using the procedure described for polyoma virus(4), or in plates, using the test procedures outlined by Takatsy(5) and Sever(6).

The test system utilized infected MK tissue culture fluid as the source of hemagglutinin, pH 7.2 phosphate-buffered saline as diluent, 1.0% human group O red blood cells, and room temperature incubation. For HI tests, 4 units of hemagglutinin were used and serum-hemagglutinin mixtures were held for 45 minutes at room temperature before addition of erythrocytes; serum was inactivated at 56°C for 30 minutes. For serum surveys, sera were screened at dilutions of 1:40 and 1:80 in tube tests, and 1:20 through 1:80 in plate tests. Antibody titers in hyperimmune mice averaged 1:160 after one inoculation, 1:1280 after 2.

In reovirus HI tests with other species sera, absorption of the serum with kaolin has been used to facilitate reading of tests(2); however, application of this procedure to mouse sera required excessive volumes of serum and resulted in a 4- to 8-fold decrease in homologous antibody; preliminary evaluation of the kaolin procedure as applied to mouse sera did not indicate that the specificity of the test was improved, and therefore kaolin absorption was not used. However, in type 3 reovirus tests using the tube method, sera were absorbed with human group O erythrocytes, since unabsorbed sera gave irregular cell patterns; 1 part of serum diluted 1:10 was mixed with 3 parts 5% erythrocyte suspension and the cells were allowed to settle overnight at 4°C. This procedure was unnecessary when plate tests were employed, since unequivocal readings could be obtained by tipping the plates and observing presence or absence of "tear-drop" slippage patterns.

Neutralization tests. Tissue culture neutralization tests were done in MK tissue cultures, using as the test dose of virus the highest dilution producing at least 3-plus cytopathic changes in 5 or 6 days (about 10^4 50% tissue culture infective doses). Sera were heated at 56° for 30 minutes and tested at a dilution of 1:10 or 1:20. A serum dilution was considered positive for neutralization if it limited the degree of cytopathic change to less than half that of the virus controls for 24 hours after the controls reached 3-plus.

Results. Isolation of reovirus type 2 from house mice. Mouse embryo tissue cultures inoculated with visceral organ suspensions from 3 wild house mice (#1220, #1223, and #1262), trapped in a single New York City apartment building, developed cytopathic changes consisting of patches and strings of small granular cells appearing after 21 to 26 days in culture. The cytopathic effects spread through the culture very slowly, requiring up to 15 days for complete destruction of the cell sheet. Cytopathic effects were reproduced on subculturing in mouse embryo tissue culture, but with only slightly shortened incubation periods; however, passage to monkey tissue culture resulted within 5 days in the distinctive cytopathic change associated with the reovirus group(7), and Giemsa-stained preparations revealed bluish intracytoplasmic masses of bizarre shape and irregular size identical to those described for the reoviruses (8). The agents were ether resistant; fluids from degenerated monkey kidney cultures agglutinated human group O erythrocytes to an average titer of 1:160; and both mouse embryo and monkey kidney tissue culture passage fluids contained complement-fixing antigen reacting with reovirus type 1 monkey antiserum and with mouse antisera against the reference strains of reovirus types 1, 2, and 3.

Preliminary HI typing of one isolate (#1223) performed in the laboratory of Dr. Leon Rosen with standard guinea pig antisera indicated a relationship to type 2; this was subsequently confirmed by Dr. Rosen in HI tests with rooster antisera to prototype strains, and with paired sera from human and bovine type 2 infections. Simultaneous tests of the latter sera with human, bovine and mouse type 2 HA antigens detected HI antibody rises of nearly identical magnitude. However, in reciprocal HI tests using mouse antisera, the type 2 strains from mice, though sharing antigens with the prototype strain (D5) of reovirus type 2, were antigenically distinguishable from the prototype(9).

Because of the long incubation period required before virus was detected in mouse embryo tissue culture, there was special need for demonstration of infection by additional tests in order to establish the wild mouse rather than the tissue culture assay system as the source of virus. The confirmatory results of reisolation and mouse antibody production tests are given in Table I. It should be noted that preparation of the pooled organ suspensions and inoculation of weanling mice for the MAP test were carried out in a field laboratory with no contact with procedures using reoviruses. Although none of the mice from which virus was isolated had significant serum antibody levels, HI or virus neutralizing activity was detected in the serum of other mice trapped in the virus-positive apartment house, as described below.

A fourth strain of Reo 2 was isolated in monkey kidney tissue culture from intestine suspension of a wild mouse trapped in a barn (Farm W.) in Montgomery Co., Maryland. This strain was serologically identical with the type 2 strains isolated from the New York mice.

Distribution of Reo 2 antibody in mice. Serologic surveys for antibody in wild and laboratory mouse colonies were done by the HI procedure using the #1223 strain as antigen (Table II). Serums from laboratory mice were consistently negative, none of 416 mice from 9 separate colonies demonstrating hemagglutination-inhibition. The occurrence of antibody in the wild mice appeared to be sharply focal in distribution. In the New York apartment house from which the virus-positive mice were trapped, 10% of mice were positive for HI antibody in the first 2 trapping periods; positive mice were found in 4 of the 6 apartment units surveyed, and on 3 of the 5 floors of the apartment house. Although positive mice were widely distributed throughout this apartment house during the first 2 trapping periods, the infection did not appear to persist, since mice trapped 4 to 6 months later from each of the 4 floors were all

TABLE I. Isolation of Type 2 Reovirus from Wild Mice.

		Detection of virus			Reovirus typing		Antibody to reovirus 2			
		Tissue culture*					Donors		Contacts†	
Source of isolate	Mouse #	ME	MK	MAP test	HI	Neut	HI	Neut	HI	CF
Mus musculus, 128 W. St. 3,‡ N.Y.C.—10% suspension of pooled viscera	1223	+ (25d)	+ (20d)	0/5§	2	2	—	trace		
Idem	1220	+ (26d)	+ (12d)	2/2	2		—	—	8/84	0/5
"	1262	+ (21d)	—	2/5	2		—	—		
Mus musculus, barn, W. farm, Montgomery Co., Maryland —10% intestine suspension	D338		+ (7d)‖	0/3	2		—		5/75	0/3

* Numbers in parentheses are the day of first appearance of cytopathic effects.
† Mice trapped in same location during comparable time period.
‡ Street number is coded(10).
§ Fractions = No. positive/No. tested.
‖ Atypical cytopathic effects seen at 7 days.

negative for antibody. Mice trapped in 11 other apartment houses on the same block during the period when positive mice were found were all negative for antibody.

A similar focal distribution was found in the farm mice. The highest incidence of antibody was in the W. farm from which the virus-positive mouse was recovered; 4 other colonies of rural wild mice yielded 1 positive animal each.

The HI antibody titers in the wild mice were as follows: 1:20 in 4 mice; 1:40 in 7; 1:80 in 1; and greater than 1:80 in 5. None of 10 of the positive wild mouse sera gave a significant reaction when tested at a dilution of 1:10 in complement fixation test against type 2 antigen.

Isolation of Reovirus type 3 from laboratory mice. Following the detection of type 2 reovirus infection in wild mice, additional strains of reovirus were obtained from laboratory mice. All of the 7 strains obtained to date have been identified as type 3 (Table III). Two of the strains were obtained from organ suspensions of normal mice; 4 from ascites-form transplanted mouse leukemias (6 were tested), and one from supernatant fluids from explant cultures of leukemic tissues of a mouse bearing a transplant of Moloney virus-induced leukemia. The isolates produced the characteristic reovirus cytopathic effects in mouse embryo and monkey kidney tissue cultures with typical cytoplasmic inclusion bodies, and when inoculated into newborn

TABLE II. HI Antibody to Reovirus 2 in Mice.

Area	Period of serum collection	No. mice positive/No. tested, by colony	Total
Wild mice—New York City, 128 W. St. 3	Aug. - Oct., 1959	4/43*	4/43
Idem	Jan., 1960	4/41	4/41
"	June - Aug., 1960	0/99	0/99
Other buildings, W. St. 3	Aug., 1959 - June, 1960	0/114, 0/20, 0/13, 0/9, 0/9, 0/7, 0/6, 0/5, 0/4, 0/3, 0/2	0/192
Wild mice—Maryland Farms	Aug., 1960 - June, 1961	5/76,* 1/68, 1/25, 0/56, 0/26, 0/7	7/258
—Grain Mills	*Idem*	1/107, 1/37, 0/45, 0/18	2/207
Laboratory mouse colonies	1959 - 1961	0/140, 0/51, 0/50, 0/39, 0/33, 0/30, 0/27, 0/23, 0/23	0/416

* Areas and periods in which virus-positive mice were trapped.

TABLE III. Isolation of Type 3 Reovirus from Laboratory Mice.

Source of isolate	Detection of virus: Tissue culture ME	Tissue culture MK	MAP test	Suckling mice*	Reovirus typing HI	Reovirus typing Neut	Serum inhibitor of reovirus 3 in donor mice HI	Neut
DBA/P retired breeders, NIH colony, 10% suspension of viscera of 12 mice.	+†		1/3	+	3	3	3/12	4/12
RFM retired breeders, Oak Ridge Nat. Lab. colony, 10% suspension of viscera of 9 mice.	—		0/2	+	3	3	7/10	7/9
Supernatant fluid from explant cultures of leukemic lymph node tissue from mouse with transplanted Moloney-virus leukemia.	+ (17d)				3	3		
Transplanted mouse leukemia, 195/S‡		+ (13d)	5/5	(+)	3			
Transplanted mouse leukemia, L1210/6MP/FU/AM‡		+ (13d)	5/5	(+)	3			
Transplanted mouse leukemia, P388/S‡		+ (13d)	5/5	(+)	3			
Transplanted mouse leukemia, 815/FU‡		+ (10d)	5/5	(+)		3		

* + = Virus recovered from tissues of suckling mice inoculated with test suspensions.
(+) = Suckling mice inoculated with test material developed characteristic picture of reovirus infection; no attempt made to recover virus from their tissues.

† Virus isolated by blind passage at 30 days.

‡ Dr. L. W. Law, suspension of tumor in ascites fluid.

mice induced the characteristic oily hair disease described for reovirus infection(3,11). Also, the tissue culture fluids infected with these isolates showed the characteristically low titers of hemagglutinin observed with type 3 strains recovered from human sources.

Reciprocal hemagglutination - inhibition tests with mouse antisera against 3 of the mouse isolates and the prototype Abney strain indicated only minor differences in antigenic composition between the mouse and human isolates.

Attempts have been made to determine the prevalence and distribution of reovirus type 3 infection in laboratory and wild mouse populations using hemagglutination inhibition and neutralization test procedures. However, serum inhibitors of both hemagglutination and infectivity were so highly prevalent among retired breeder laboratory mice, including mice of specific-pathogen-free and, in some instances, germ-free colonies, that the possibility of non-specific serum inhibitors must be considered, particularly since the titers encountered were generally low (1:20 to 1:80). Attempts to evaluate the significance of the serum inhibitors by correlative complement fixation and virus isolation studies are in progress. Serum inhibitor of type 3 hemagglutination and infectivity has also been found in wild mice, but less frequently than in the majority of laboratory mouse colonies.

Discussion. The serologic survey data presented here suggest that type 2 reovirus infection may not be an indigenous infection of the mouse, but that the infections observed may have been acquired from contact with other species. This was suggested by the low frequency of antibody in the positive foci and by the apparent disappearance of infection from the New York focus. If this supposition is correct, it would appear likely that the New York mice acquired the infections from humans and that the Maryland farm mice may well have acquired the infection from cows. However, it is perhaps significant that the strains isolated from the 2 different areas were of the same subtype. The possibility must be considered that the infected mice could serve as a source of infection for humans and other animals.

Although the question was raised that the serum inhibition of type 3 reovirus may be due to non-specific inhibitors, the type 2 serologic results would appear to be specific, since the occurrence of antibody correlated well with the presence of mice infected with the virus. The possibility that some of the type 2 reactions may represent cross reactions from infection with other reovirus types must be considered; however, no correlation has been observed between the presence of type 3 inhibitor and inhibition of type 2 in HI tests of individual mouse sera, and tests with sera of immunized mice indicate that the HI and neutralization procedures give only minimal cross reaction.

The finding of reovirus type 3 virus in 5 of 7 transplanted mouse leukemias suggests that this virus may be a relatively frequent contaminant of mouse tumors. Also, we have identified the oncolytic virus isolated by Nelson(12) from a transplantable ascites tumor as a strain of reovirus type 3.

Summary. Using virus isolation and serologic survey procedures, the occurrence of natural reovirus infection in mice has been detected. Four isolates of reovirus type 2 were recovered from urban and rural wild mice; 7 strains of reovirus type 3 were isolated from normal laboratory mice and transplant-induced mouse leukemias. Antibody to type 2 was found focally distributed in wild mice, but was not encountered in laboratory mice. A low titer serum inhibitor of type 3 HA and infectivity, possibly representing specific antibody, was prevalent in laboratory mice.

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Effects of Gonadotrophins and Androgens on the Sex Organs of the Estrogen-Treated Male Rat. (26949)

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In an effort to use estrogen-treated immature male rats for LH assays it became apparent that the ventral prostate response to crude and purified preparations was quite different. Data obtained in examining these differences are here presented.

Methods. In the first series of experiments, 17-18 day old male rats (Charles River Lab.) were given a single subcutaneous injection of 5 μg of estradiol dipropionate in peanut oil. In the second series, 20-21-day-old Sprague Dawley rats were injected with 10 μg of estradiol dipropionate in peanut oil. The animals were fed *ad libitum* with Purina Lab Chow.

Subcutaneous injections of the materials to be tested were begun 3 days after administration of the estrogen. All materials were given for 4 successive days, except testosterone propionate which was given as a single injection. The volume of material injected was 0.25 ml. The following preparations were used:

1. Estradiol dipropionate, Ciba, in sesame oil, diluted with peanut oil to desired concentration,
2. Testosterone propionate, Ayerst, diluted to volume with peanut oil,
3. Luteinizing Hormone (LH) in 15% gelatin—0.2% phenol,*
    a. Equine, Armour Co., Lot #377-279
    b. Ovine, Armour Co., Lot #227-80
    c. Ovine, DEAE—LH (A neutral 75% ethanol precipitate of an ammoniacal-alcohol extract of acetone dried glands was put on a diethylaminoethyl cellulose column in 0.005 M ammonium hydroxide-ammonium acetate buffer at pH 9.8. The first fraction obtained after initiating gradient elution with 0.1 M ammonium acetate was dialyzed, lyophilized and used in these experiments.
4. Acetone-dried ovine pituitaries, ground to 60 mesh, and crude extracts of ovine pituitaries, were suspended or dissolved in 15% gelatin—0.2% phenol,
5. Testosterone (free alcohol), Δ 4-androstene-3,17 dione, progesterone, 17 *a* hydroxyprogesterone, dehydroepiandrosterone and pregnenolone (G. D. Searle and Co.) were dissolved in peanut oil.

All animals were sacrificed 7 days following administration of estrogen. Body weight was measured. The right testis, ventral prostate, and seminal vesicles were dissected free, blotted on paper, and weighed.

Results. Series I. The 25-26-day-old rats (Charles River Lab.) had ventral prostates that weighed 50.2 (± 2.1) mg/100 g rat (n-20). When injected subcutaneously with 5 μg of estradiol dipropionate at 17-18 days of age and autopsied 7 days later, rats of this strain had ventral prostates that weighed 28.5 (± 1.7) mg/100 g rat (n = 39).

Data illustrated in Fig. 1 demonstrate the effect of testosterone propionate (TP) on the ventral prostate of the estrogen-treated immature rat. A linear relationship between the logarithm of dose of TP and ventral prostate size (mg/100 g rat) was found to exist, such that a 10-fold increase in TP dose resulted in an average increase in ventral prostate size of 41.5 mg/100 g rat. The minimum dose of TP required to significantly enlarge the ventral prostate of these animals varied from 10 to 20 μg. Ventral prostates heavier than 100 mg/100 g rat were obtained with large doses of TP.

Data indicated that the responses of the ventral prostate to Armour equine LH 377-279 and crude sheep pituitary extracts were similar (Fig. 1). The increase in ventral prostate size following a 10-fold increase in dose of these pituitary extracts ranged from 25.9 to 38.2 mg/100 g rat. Ventral prostate weights between 70 and 115 mg/100 g rat were repeatedly observed following administration of relatively large doses of sheep pituitary powder and crude extracts thereof.

In contrast to TP and crude pituitary extracts, rats given increasing doses of ovine DEAE-LH showed relatively small increases in ventral prostate weight (average slope

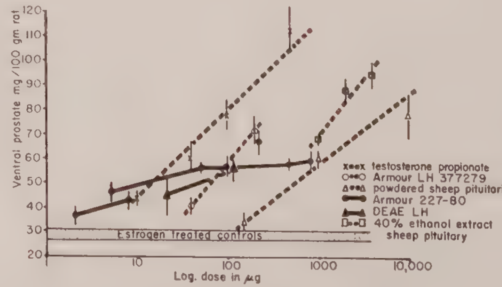

FIG. 1. *Series I*: Effect of testosterone propionate and pituitary extracts on ventral prostate weight of estrogen-treated immature rat.

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* Armour LH 377-279 was reported to have 5 times the potency of Armour LH 227-80 when assayed on the basis of response of the ventral prostate of hypophysectomized rats rested 7 days following operation. LH 377-279 was, however, only 15-20% as potent as LH 227-80 when assayed by Dr. Albert Parlow using ovarian ascorbic acid depletion in pseudopregnant rats as the end point. DEAE-LH was approximately 30% as potent as LH 227-80 when assayed in hypophysectomized male rats rested 2 days following surgery, and when assayed by the ovarian ascorbic depletion technique.

TABLE I. *Series II.* Effects of LH, Pituitary Powder, and Various Steroids on Weight of Reproductive Organs of Estrogen-Treated, Immature Male Rats.

Material	Total dose, µg	n	Body wt, g ± S.E.	Seminal vesicle	Ventral prostate	R. testis	
				mg/100 g ± S.E.			
Controls	0	8	70.0 ± 3.4	29.5 ± 1.4	19.2 ± .8	207 ± 15	
Armour LH, Lot 227-80	5	10	73.8 ± 2.8	23.8 ± 1.7	40.5 ± 3.1	300 ± 10	**
	10	5	74.2 ± 1.1	27.4 ± 1.3	47.7 ± 7.3	312 ± 14	**
	20	11	75.8 ± 2.0	30.3 ± .9	57.9 ± 3.3	293 ± 7	**
	40	11	70.1 ± 1.9	28.8 ± 5.3	55.0 ± 5.0	287 ± 18	*
	800	6	70.0 ± 2.8	40.7 ± 5.3	76.7 ± 2.3	349 ± 15	**
Sheep pituitary powder	200	6	71.3 ± 2.7	27.3 ± 1.7	28.2 ± 1.7	246 ± 12	NS
	300	6	72.8 ± 3.3	35.9 ± 3.9	40.9 ± 4.6	285 ± 14	*
	1200	6	77.0 ± 2.6	34.7 ± 3.5	61.0 ± 8.3	358 ± 27	**
	2000	5	74.0 ± 1.6	31.6 ± 2.8	56.3 ± 6.9	329 ± 19	**
	20000	6	70.8 ± 1.5	46.1 ± 3.6	84.4 ± 7.4	370 ± 14	**
	32000	8	75.9 ± 2.0	55.0 ± 3.7	90.5 ± 6.2	371 ± 17	**
Testosterone	100	12	75.6 ± 1.3	34.4 ± 2.3	37.3 ± 2.9	206 ± 6	NS
	400	6	73.7 ± 2.6	40.5 ± 1.5	52.7 ± 7.1	213 ± 9	NS
	800	6	72.7 ± 4.1	63.4 ± 9.7	63.2 ± 2.8	230 ± 10	NS
	1600	9	72.0 ± 1.6	77.0 ± 5.5	82.6 ± 3.4	272 ± 23	NS
	10000	7	76.1 ± 1.8	146.1 ± 3.5	107.4 ± 3.1	284 ± 6	*
Δ_4-Androstene-3,17 dione	100	6	73.0 ± 1.3	33.6 ± 9.1	45.9 ± 6.1	219 ± 17	NS
	400	6	75.5 ± 1.7	26.7 ± .9	54.7 ± 5.2	262 ± 33	NS
	800	6	74.3 ± 1.3	33.9 ± 1.9	68.7 ± 6.3	222 ± 7	NS
	1600	6	73.3 ± 2.0	54.2 ± 4.9	87.8 ± 4.6	245 ± 13	NS
	10000	6	73.1 ± 1.9	107.0 ± 4.6	115.2 ± 11.7	297 ± 10	*
17α hydroxyprogesterone	2000	6	77.2 ± 1.0	24.2 ± 1.3	32.9 ± 5.5	231 ± 34	NS
	10000	6	74.3 ± 1.3	32.1 ± 1.9	67.4 ± 4.2	282 ± 15	*
Progesterone	2000	5	74.8 ± 2.5	26.8 ± 2.0	41.9 ± 2.4	223 ± 5	NS
	5000	6	74.7 ± 1.0	27.3 ± 2.2	53.3 ± 7.6	231 ± 14	NS
	20000	6	71.8 ± 2.0	26.3 ± 2.3	66.6 ± 6.4	245 ± 18	NS
Pregnenolone	1000	8	72.9 ± 1.4	30.3 ± 1.7	35.0 ± 2.4	258 ± 8	*
	3000	6	71.5 ± 3.9	27.7 ± 2.0	43.8 ± 3.9	297 ± 17	*
	6000	7	76.4 ± 2.0	32.3 ± 1.4	52.1 ± 3.3	277 ± 6	*
	15000	10	72.5 ± 1.3	26.0 ± 1.6	51.4 ± 3.2	281 ± 8	**
Dehydroepiandrosterone	800	6	76.8 ± 3.5	28.2 ± 1.5	29.4 ± 4.7	248 ± 3	NS
	1600	6	77.7 ± 2.8	30.0 ± .7	35.8 ± 2.8	251 ± 11	NS
	2100	6	73.0 ± 2.8	25.7 ± 2.8	45.2 ± 8.7	279 ± 8	NS
	8400	6	76.8 ± 3.7	47.7 ± 3.9	70.5 ± 5.4	306 ± 11	*

Statistical significance: * $p < .05$; ** $p < .01$; *** $p < .001$; NS—not significant.

9.2). A similar result was obtained with Armour Ovine LH 227-80 (slope 13.4) (Fig. 1). Furthermore, approximately 50 µg of Armour LH 227-80 produced ventral prostate weights of 55-65 mg/100 g rat and increasing the dose to 1.0 mg did not induce further enlargement.

Series II. Data in Table I again demonstrated that increasing doses of Armour Ovine LH 227-80 induced smaller increments in ventral prostate weight (slope 15.7) than did powdered sheep pituitaries (slope 26.7), testosterone (slope 36.0), androstenedione (slope 36.9), progesterone, 17 α hydroxyprogesterone, or dehydroepiandrosterone. Pregnenolone caused ventral prostate enlargement with dose-response characteristics similar to those of Armour LH 227-80 (slope 14.6). Statistical comparisons of these slopes are presented in Table II.

For each material assayed, the weight of the ventral prostate (mg/100 g rat) was plotted against the logarithm of the dose and extrapolated to obtain the minimum effective dose (amount of material required to produce a ventral prostate weighing 21.6 mg/100 g rat). The data were then replotted (Fig. 2A) as mg ventral prostate/100 g rat against the logarithms of multiples of the minimum effective dose (actual doses divided by minimum

TABLE II. *Series II.* A Comparison of Dose-Ventral Prostate Response Slopes Following Administration of Pituitary Extracts and Androgenic Steroids Utilizing the Chi-Square Test for Parallelism.

	Slope (b)	Variance ($\sigma^2 b$)	χ^2	P	Parallelism
Testosterone	36.02	5.861			
vs					
Armour 227-80	15.65	9.036	27.85	$<.001$	Not parallel
Sheep pit. powder	26.70	11.237	5.08	$<.05 >.02$	" "
Androstenedione	36.92	20.902	.03	$<.99 >.98$	Parallel
Pregnenolone	14.62	12.580	24.83	$<.001$	Not parallel
Armour 227-80					
vs					
Sheep pit. powder			6.02	$<.02 >.01$	Not parallel
Pregnenolone			.05	$<.99 >.98$	Parallel
Sheep pit. powder					
vs					
Pregnenolone			6.13	$<.02 >.01$	Not parallel

effective dose). Similarly, where significant enlargement occurred, weights of seminal vesicles (Fig. 2B) and testes (Fig. 2C), were plotted against the logarithms of multiples of the minimum effective dose as calculated from the ventral prostate data. Thus the abscissa of the plot was made the same for all materials assayed and for all organs.

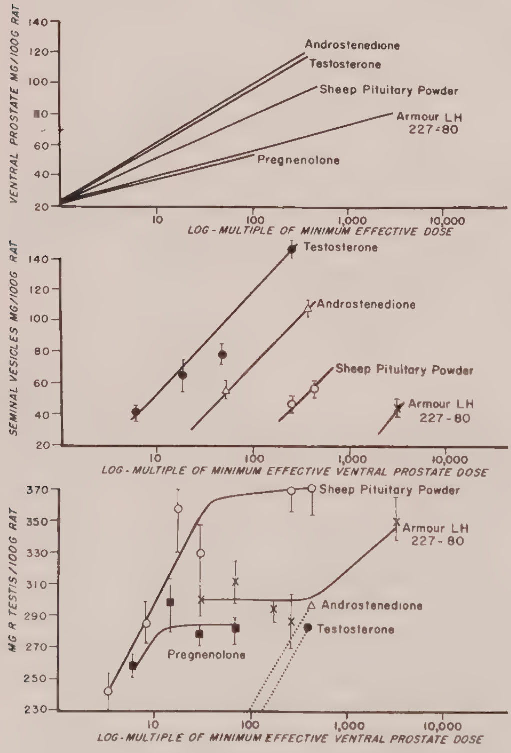

FIG. 2. *Series II*: Effects of graded doses of pituitary extracts and androgenic steroids on sex organs of estrogen-primed rat. (See text for explanation of abscissa.)

The dose of testosterone capable of inducing minimal but definite growth of the seminal vesicles was only 2 to 3 times that necessary to produce minimal growth of the ventral prostate (Fig. 2B). Ten to 20 times as much androstenedione was required to stimulate the seminal vesicles as was needed to stimulate the ventral prostate, while for sheep pituitary powder 100-110 times, and for Armour LH 227-80, 1000-2000 times as much was required. Progesterone, 17 α hydroxyprogesterone, pregnenolone, and dehydroepiandrosterone either did not cause enlargement of the seminal vesicles or did so only at high dose levels.

The threshold dose for enlargement of the testes was similar to the threshold dose for enlargement of the ventral prostate when sheep pituitary powder, Armour LH 227-80 or pregnenolone were administered, but was considerably greater when the other androgenic steroids were given (Fig. 2C).

Discussion. Several patterns of response were observed when increasing doses of pituitary extracts or steroids were administered to immature, male rats previously treated with estradiol dipropionate. One response, seen after administration of Armour LH 227-80 or pregnenolone, was characterized by small increments in ventral prostate weight, no increase in seminal vesicle weight except with large doses, and significant enlargement of the testes with doses barely affecting the size of the ventral prostate.

Another response, occurring with administration of whole pituitary powder, was typified by relatively large increments in ventral prostate size and no increase in seminal vesicle weight until large doses were administered. Similar although not identical effects were noted with Δ 4-androstene-3,17 dione, 17 α hydroxyprogesterone, and dehydroepiandrosterone. These steroids did not increase testes weight except with large doses. Whole pituitary powder, probably because of its FSH and LH content, induced enlargement of the testes even at doses that caused only minimal increase in ventral prostate weight.

A third pattern of response occurred with testosterone which caused nearly coincident stimulation of the seminal vesicles and ventral prostate with relatively great dose-response slopes, and no increase in testes weight except with large doses. The synergistic action of testosterone and estrogens in affecting growth of the seminal vesicles has been reported(1). It would appear that the other androgenic steroids administered in this study, and the endogenous androgens elaborated by the rat when stimulated by ovine gonadotrophins, did not synergize with the administered estradiol dipropionate.

Purified equine and ovine LH preparations produced different ventral prostate dose-response relationships, the equine material having a greater slope than the ovine LH (Series I). The same phenomenon has been found in the hypophysectomized rat and has been explained by the longer biological half-life of the equine LH(2). A similar mechanism, slow absorption of crude pituitary extracts and rapid absorption of purified LH preparations, may account for the differences in dose-response slopes of these two materials. However, use of a gelatin vehicle should have minimized any potential differences in rate of absorption. Moreover, attempts to further delay absorption of purified LH preparations with an oily vehicle did not enhance their action(3).

The finding that a highly purified LH preparation caused only a doubling of ventral prostate weight in hypophysectomized rats tends to substantiate the data presented here(4). The hypothesis that LH is responsible for production of a weakly androgenic substance such as pregnenolone, while crude pituitary extracts lead to formation of a more potent androgen such as androstenedione, is appealing and compatible with the data presented, but is not substantiated by it. Corroboration of the data presented here, and thus circumstantial support for this hypothesis, can be found among previously reported observations. LH has been shown to maintain spermatogenesis and testicular weight in hypophysectomized rats with doses barely affecting seminal vesicle or ventral prostate size(5), while testosterone propionate did so only at dose levels that were distinctly androgenic(6). Similar observations have been made on the hypophysectomized mouse(7). Pregnenolone has been reported to be more potent in maintaining spermatogenesis and testicular weight in hypophysectomized or estrogen-treated rats than either testosterone, androstenedione, or progesterone(8,9).

The factor (or factors) present in crude pituitary extracts making them more effective than purified LH in stimulating growth of the ventral prostate is unknown. Theoretically, this postulated factor could directly enlarge the ventral prostate, augment the response of the ventral prostate to androgen, or facilitate androgen production by the testes. There are conflicting data in the literature concerning these possibilities. No effect on weight of the ventral prostate, nor augmentation of LH or testosterone effects on weight of the ventral prostate weight, has been noted with FSH(10,11,12) or ACTH(11,13). Growth hormone has been reported to enlarge(11) and to have no effect(14) on the ventral prostate of the hypophysectomized-castrated rat. No effect of growth hormone on the ventral prostate of the castrated rat has been noted (13). Growth hormone has been reported to have little(13) or no(14) effect on the response of the ventral prostate to testosterone. Growth hormone has been reported to augment LH effects on the ventral prostate in one strain of hypophysectomized rats but not another(12).

The reported effects of prolactin on the ventral prostate have also been variable. Prolactin had no effect on the ventral prostate of the hypophysectomized-castrated rat(11,14) and only minor effect on the ventral prostate of the castrated rat(13). Prolactin had been noted to augment the effect of testosterone on the ventral prostate of the hypophysectomized rat(15), but only questionable augmentation has been noted in the hypophysectomized-castrated animal(14). Little(13) or no(16) augmentation was noted in castrated rats. A synergistic effect of LH and prolactin on the ventral prostate has been noted in one strain of hypophysectomized rats(12,17) but not in another(12). It could not be shown that prolactin augmented the ventral prostate response to urinary gonadotrophins (16). No consistent augmentation of LH effects in the estrogen-treated rat could be shown with ACTH, TSH, FSH, or various combinations of growth hormone and prolactin(3).

Summary. The effects of various pituitary extracts and androgenic steroids on the sex organs of the estrogen-treated immature male rat were studied. Testosterone induced growth of the seminal vesicles and ventral prostate with similar threshold-doses and dose-response slopes for both organs, but did not cause testicular enlargement except at high dose levels. Purified ovine LH and pregnenolone induced growth of the ventral prostate but the dose-response slope was considerably less than that obtained with testosterone, androstenedione, 17 α hydroxyprogesterone, progesterone, dehydroepiandrosterone or crude pituitary extracts. Unlike testosterone, neither purified LH nor pregnenolone induced growth of the seminal vesicles except at very high dose levels, but both caused enlargement of the testes at doses that barely effected prostate size.

I am grateful to Dr. E. B. Astwood for helpful advice and the use of his laboratory at New England Center Hospital, Boston, Mass., where these experiments were begun. This work was supported, in part, by U. S. Pub. Health Grant A3758.

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Cataract Development in Rats Fed Commercial Quercetin.* (26950)

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The metabolic fate of quercetin (3,3′,4′,-5,7-pentahydroxy flavone) in animals and its apparent lack of toxicity have been investigated by several research groups (1-5). Recently it has been discovered in this laboratory that a dose of 20 mg or more of commercially purchased quercetin will produce cataracts in eyes of a significant percentage of laboratory rats when administered orally. The effects of purified quercetin and of the impurities found in the commercial quercetin have also been studied.

Methods. Male albino rats, Holtzman strain, 2 months old, were fed a vitamin B complex test diet (Nutritional Biochemicals Corp., Cleveland, Ohio), with complete vitamin fortification for at least one month before use of quercetin. This diet was also continued throughout the test periods. Weighed amounts of the commercial quercetin (Nutritional Biochemicals Corp.) were suspended in U.S.P. syrup, Spec. Grav. 1.313, and administered to each rat by stomach tube.

Results. When a suspension containing 100 mg of the commercial quercetin was put into the stomach of each of 10 rats (3 months old) as a one-dose administration, one rat died within 7 days. Of the remaining 9 rats, 4 developed one cataract each within 4 weeks after receiving the single dose of the commercial quercetin. Microscopic examinations of eye sections confirmed the presence of the cataracts. Vesicular degeneration was noted in the posterior lens capsule and in the body of the lens. An uneven eosinophilia was noted throughout the capsule and body of the lens after staining with hematoxylin and eosin. No changes were noted in the retina or the epithelial area which in non-albino animals is normally pigmented. Two additional rats of this group developed cataracts within 10 weeks after the single dose of quercetin was given. Twelve control rats (6 given syrup, 6 synethic diet only, except that quercetin was not administered) were completely normal after the same length of time.

When a total of 20 mg of the commercial quercetin was administered by stomach tube to each rat of a second group (6 rats, 5 months old) in doses of 5 mg daily for 4 days, 3 of the 6 rats developed cataracts within 6 weeks after the initial dose. No cataracts were found in the controls (6 syrup, 6 synthetic diet only). When a total of 100 mg of the commercial quercetin was put into the stomach of each rat of a third group (8 rats, 6 months old) in doses of 25 mg daily for 4 days, 4 of the 8 rats developed cataracts after 4 weeks. A typical cataract development in this group is shown in the photograph (Fig. 1a) and compared with a normal eye in the other photograph (Fig. 1b).

Using a silicic acid column, chromatographically pure quercetin was obtained. When this pure, crystalline quercetin was administered by stomach tube to rats in

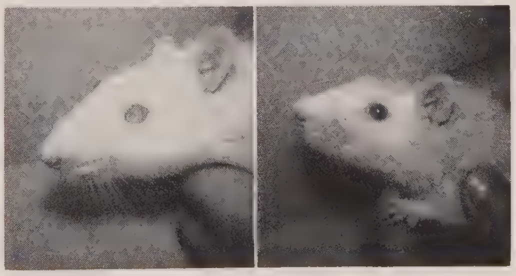

FIG. 1.

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* This work is supported by N.I.H. research grants.

doses (25 mg/day/4 days) as previously described for the experiments using commercial quercetin, no cataracts were found in a group of 6 rats after 3 months following the administration of quercetin.

The mixture of impurities separated from the quercetin on the silicic acid column was eluted as a group and fed to 2 rats. Within 2 weeks, 1 of these 2 rats already exhibited the beginning of cataract development.

Work is now in progress on separation of the 8 or more individual compounds present as impurities in the commercial quercetin and on testing the effect of each compound on development of cataracts.

Summary. When 20 mg or 100 mg of commercial quercetin suspended in U.S.P. syrup was put into the stomach of a rat, either in a single dose or in 4 daily doses of 5 mg or 25 mg respectively, 50% of the animals so treated developed a cataract in one eye within 10 weeks following receipt of the quercetin. No bilateral cataracts were observed. Chromatographically pure quercetin did not cause cataracts under similar conditions.

We are grateful to Dr. W. Marvin Davis, Univ. of Oklahoma School of Pharmacy for many helpful suggestions, and to Mr. Russell Allen, NIH Post Sophomore Medical Research Fellow, for microscopic examination of the eye sections.

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Connective Tissue Defect in the Chick Resulting from Copper Deficiency.* (26951)

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Since the observations of Hart *et al.* (1), the primary physiological role of copper has been assumed to be concerned with hemopoiesis. A function in connective tissue metabolism has been suggested by bone deformities that occur in dogs (2) pigs (3) and chickens (4), and by the subcutaneous hemorrhage and abdominal hernias observed in newborn rats (5) when these animals were deprived of dietary copper. Gallagher (4) was unable to produce copper-deficiency anemia in chicks but recently Hill and Matrone (6) have produced anemia in presence of adequate iron. The purpose of the present study was to investigate other aspects of the pathology of copper deficiency in chicks.

Methods. Crossbred Vantress X White Rock chicks were kept in battery brooders which had been coated with an epoxy resin. Eight 4-week trials were run using 10 or 20 chicks per group. The basal diet had the following composition: Non-fat milk solids 55.0 g, sucrose 35.3 g, soybean oil 5.0 g, glycine 1.5 g, L-arginine HCl 1.0 g, DL-methionine 0.5 g, $CaHPO_4$ 1.0 g, NaCl 0.5 g, choline Cl 0.2 g, $MnSO_4 \cdot H_2O$ 31 mg, $FeSO_4 \cdot 7H_2O$ 25 mg, KI 0.3 mg. A vitamin

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TABLE I. Gross Pathology of Copper Deficiency.

Observations	Basal	Basal + 5 ppm Cu
Avg wt, g	244 ± 6*(86)†	388 ± 7 (77)
Mortality, %	23 (112)	1.3 (78)
Packed red cell vol, %	28.5 ± .5 (60)	31.5 ± .5 (52)
Leg weakness, %	31 (90)	2 (60)
Bone deformity or perosis, %	21 (90)	2 (60)
Subcutaneous hemorrhage, %	47 (90)	2 (60)
Internal hemorrhage at necropsy, %	78 (40)	0

* Stand. error of mean. † No. of chicks upon which observation was based.

supplement (7) was added.† By analysis this diet contained about 0.8 ppm of copper and the control diet was supplemented with 20 mg of $CuSO_4 \cdot 5H_2O$ per kg. Feed in resin-coated troughs and distilled water in glass fountains were supplied *ad libitum*. At the end of the fourth week blood was drawn from the ulnar vein for hematocrit determination and tissues were saved for histological examination. In some cases tissue was taken immediately after death that resulted from internal hemorrhage. Aortae were fixed in neutral formalin, embedded, sectioned and stained according to standard procedures (8).

Results and discussion. A summary of the gross pathology observed in the copper deficient chicks is presented in Table 1. Although the severity of depletion varied from trial to trial, on the average chicks that consumed the copper deficient diet weighed significantly ($P < 0.01$) less at 4 weeks of age. There was only a 10% decrease in average hematocrit value, but the difference was statistically significant ($P < 0.01$).

One of the early symptoms of the deficiency was a tendency for the chicks to squat or to exhibit an unsteady gait. Although it was difficult to make a clear distinction at all stages between this leg weakness and the condition commonly described as perosis, it was evident that severely depleted chicks frequently became completely paralyzed without showing either slipped tendon or bone deformities. Approximately 30% of the copper-deficient chicks exhibited some degree of leg weakness or paralysis. Occasionally there was associated with these symptoms a bone deformity characterized by sharp bending at the proximal end of the metatarsus. In general, the bones were more fragile than normal.

The most spectacular symptom was the widespread occurrence of both subcutaneous and internal hemorrhage. Subcutaneous hemorrhages became evident in a high percentage of the birds by the end of the third week and was most commonly observed on the thighs and breast. Essentially no hemorrhages occurred in chicks that consumed the copper-supplemented diet.

There was a rather high mortality rate (23%) among the copper deficient chicks and a large proportion of the deaths could be attributed to internal hemorrhage. Of 40 chicks necropsied up to 6 weeks of age 78% showed massive blood clots in the pericardial sac or in the peritoneal cavity, providing evidence for a defect in the atria, aorta and other major vessels.

The aorta was chosen for histological study and photomicrographs of selected sections are shown in Figs. 1-6. The section shown in Fig. 1 was taken from a copper deficient chick soon after death. There was a massive hemorrhage in the peritoneal cavity and the aorta was discolored from hemorrhage within the vessel wall. The walls of the aorta were greatly thickened, the lumen was extremely small, and dissect-

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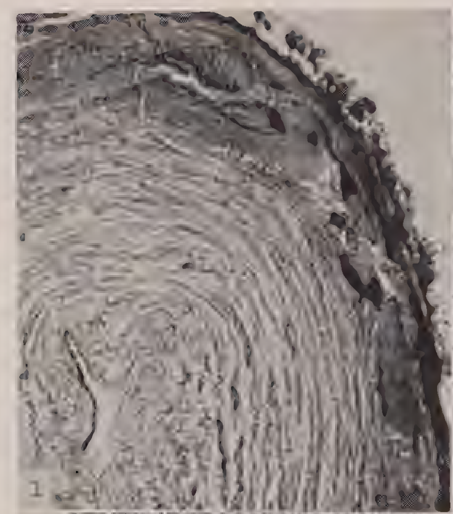
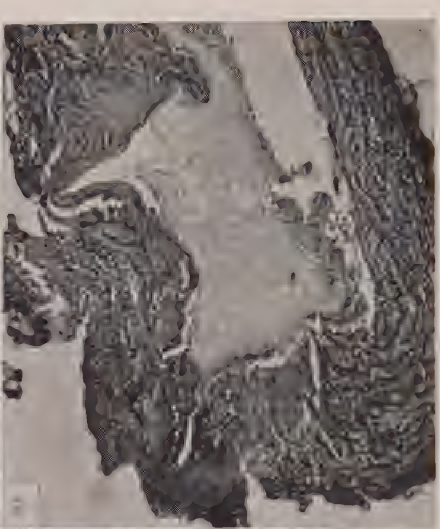
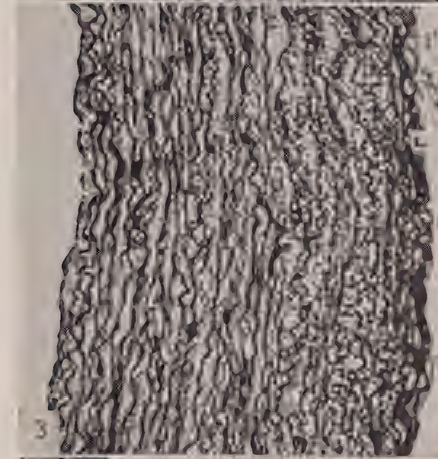
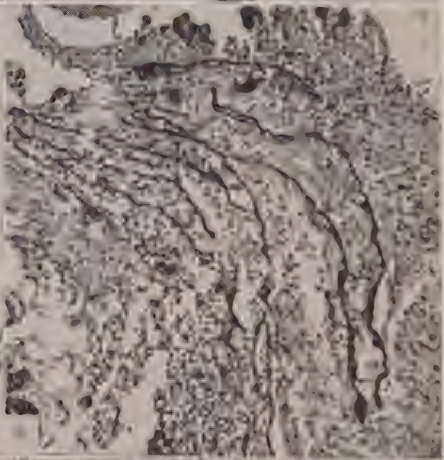
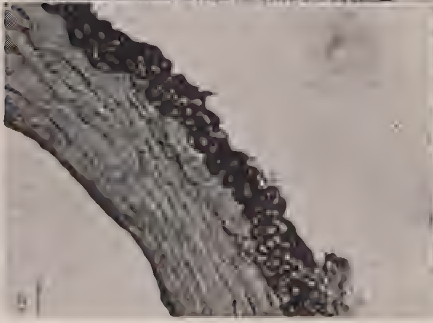
†Folacin was supplied by American Cyanamid Co., biotin by Hoffmann-LaRoche, the other vitamins by Merck and Co., methionine by Dow Chemical Co., arginine by General Mills, Inc. and glycine by Monsanto Chemical Co.











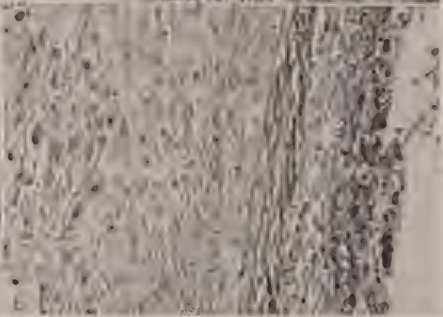


FIG. 1. Cross-section of aorta from copper-deficient chick showing dissecting aneurysm and thickening of the medial wall. Weigert × 45.

FIG. 2. Cross-section of deficient aorta showing rupture of the wall just distal to the arch and attempted repair of a previous break. Weigert × 45.

FIG. 3. Cross-section of control aorta showing normal elastic fibers in an area near arch. Weigert × 150.

FIG. 4. Cross-section of deficient aorta. Note fragmentation and focal breaks in elastic fibers and wide separation of laminae. Weigert × 150.

FIG. 5. Cross-section of abdominal aorta from control chick. Weigert × 150.

FIG. 6. Cross-section of abdominal aorta from deficient chick. Note sparsity of elastic fibers and accumulation of non-elastic tissue in media. Weigert × 150.

ing aneurysm was clearly evident. The aortic section shown in Fig. 2 was taken from a chick that died from pericardial hemorrhage. Grossly the aorta was enlarged and dark in color. The histologic section taken just distal to the aortic arch showed essentially complete rupture of the vessel wall. The elastic fibers were disrupted and attempted repair of the damage at one point was evident.

A section of a control aorta just distal to the arch (Fig. 3) shows numerous elastic fibers throughout the media. In contrast, the elastic laminae in the deficient aorta (Fig. 4) were fragmented, showed focal breaks and were widely separated by material that stained green by the Gomori trichrome method and was probably collagen or a collagen-like material. The amount of this material varied with degree of damage and was not present in all copper deficient aortae observed. Figs. 5 and 6 show abdominal aortae of control and deficient chicks. In this area the elastic fibers in the control aorta were concentrated in the outer portion of the vessel wall. The wall of the deficient aorta was greatly thickened and the elastic fibers were only barely visible.

The lesions described here suggest that copper plays a role in connective tissue metabolism. The exact nature of the derangement in copper deficiency is not known but the histologic observations suggest a defect in elastic tissue and an accumulation of an unidentified material in the interlaminar spaces.

Inasmuch as mortality from internal hemorrhage was high whereas the anemia produced was rather mild, it is evident that in this species the role of copper in connective tissue metabolism is more important for survival than its role in hemopoiesis. Hemorrhage appears to be related to the integrity of the vessel walls rather than to a coagulation defect. Although a controlled study of coagulation was not made, grossly the blood clotted in a normal manner. Whether or not the leg weakness was due to a connective tissue defect is not clear, but the deformities of the bones were no doubt related to the connective tissue defect.

Aortic lesions similar to those described above have been observed in copper deficient swine (9). A high proportion of the animals died of aortic rupture and sections of the aorta showed dissecting aneurysm and focal breaks in the elastic laminae.

Summary. Chicks reared for 4 weeks on a modified non-fat milk diet deficient in copper developed mild anemia, leg weakness, bone deformities and a high incidence of subcutaneous hemorrhage. Mortality amounted to 23% and most deaths resulted from internal hemorrhage associated with rupture of major vessels. Histologic studies suggest a derangement of connective tissue metabolism with the major defect in elastic tissue.

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A Critique of the Plaque Assay Technique in Bottle Cultures.* (26952)

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Since its introduction Dulbecco's plaque technique (1) has been widely used for obtaining viral clones. Conditions for assuring that virus derived from a single plaque represented progeny of a single infective unit were investigated and defined by Dulbecco and Vogt (2). Modifications of the technique (4,5,6) have been proposed and adopted in many laboratories. In these instances control studies of the purity of virus isolated from plaques such as described by Dulbecco and Vogt were not mentioned.

During preliminary work with plaque purification of measles virus in bottle cultures the agent was demonstrated 11 days after inoculation in the small amount of fluid that collects in the vessel. This observation suggested that agar surfaces in bottle cultures might become widely contaminated with virus. If so, the reliability of this technique as applied to the cloning of viruses would obviously be in doubt. Accordingly, an investigation was undertaken to determine, using polio virus and HeLa cells, the distribution of virus in various regions of the system.

Methods and materials. HeLa Cells. HeLa cells from stock cultures were inoculated in milk dilution bottles measuring 40 × 40 × 105 mm, using Eagle's minimal essential medium (3) with 10% inactivated calf serum. Infection was carried out when the monolayer was confluent, usually on the fourth day.

Monkey Kidney Epithelial Cells. Monolayer cultures in screw-capped tubes were obtained from Microbiological Associates, Inc. They were maintained on medium 199 with 2% inactivated calf serum.

Polioviruses. Most experiments were carried out with a stock culture of poliovirus type I, Brunhilde strain. In some experiments poliovirus type III, Leon strain, was also used.

Plaque Technique. Stock virus of known titer was diluted to yield from 1 to 30 plaques. Bottle cultures were inoculated with 0.3 ml of the suspension and incubated at 36° C for 1 hour on a shaking machine moving at slow speed. Overlay consisted of Eagle's minimal essential medium with 5% inactivated calf serum in 1.5% agar. Cultures were inverted and incubated at 36° C. Care was exercised to maintain the cultures in the same position throughout the experiment.

Results. Presence of Virus on Agar Surface. To determine whether virus could be recovered from the agar surface of bottle cultures exhibiting a small number of plaques, points were arbitrarily marked on the glass at the time of agar overlaying. A cotton swab was touched against the agar lightly at these points on successive days. The swab was then moistened in culture medium; fluid was expressed in monkey kidney cell cultures which were incubated at 36°C for 7 days. No poliovirus was detected on the agar surface by this method prior to the appearance of plaques. When a plaque directly underlay the surface point tested, virus was recovered at the surface on the same day the plaque became visible. When a plaque was not directly under the joint tested but within 0.5 cm, virus was usually recovered one or 2 days after the plaque was first seen. At greater distances from the edge of a plaque, virus was recovered twice on the 2nd day after appearance of the plaque in 13 tests (Table I).

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* Supported in part by Research Grant from Nat. Inst. of Allergy and Infect. Dis., Nat. Inst. Health, P.H.S., U. S. Department of H.E.W.

TABLE I. Recovery of Poliovirus Type I from Agar Surface of Bottle Cultures.

Distance from point tested to edge of nearest plaque (cm)	No. of points tested	No. of positive cultures
.5-.9	6	1
1 or greater	7	1

The glass sides of 3 bottles were found to be free of virus. The agar surface of these was washed with 2.0 ml of sterile culture fluid. Virus recovered from the surface in this manner totalled $10^{2.1}$, $10^{2.6}$ and $10^{2.6}$ pfu from bottles with 2, 6, and 9 plaques, respectively.

Presence of Virus on Exposed Glass. To assay for the presence of poliovirus on the glass surfaces of the bottles 2.0 ml of culture fluid was run over the wall opposite the overlay, taking care to prevent the medium from coming in contact with the agar surface. Recovery of poliovirus was determined by inoculating the total volume of fluid into monkey kidney cultures or quantitated by plaque titration on HeLa cells. Poliovirus was thus detected in 14 of 24 bottles tested (58%).

The time and rate of appearance of poliovirus on the glass surface was assayed by titrating washings obtained 30 minutes after adding the agar overlay and each day thereafter through the fourth day. Results for 2 bottle cultures are shown in Table II. Absence of virus immediately following inoculation and increase of the agent following its first appearance indicate that it arose as a result of multiplication within the system.

No correlation was established between the number of plaques or their location and presence or degree of contamination of the glass walls.

Purity of Virus Derived from Plaques. When bottles were simultaneously infected with type I and type III polioviruses, both agents were subsequently recovered from the glass. Accordingly, mixed infections were carried out and the purity of virus isolated from a number of plaques was determined in the following manner.

Bottle cultures of HeLa cells were inoculated with sufficient poliovirus type I and type III to yield approximately 5 to 15 plaques of each. Five days later virus isolation from several of the resulting plaques was effected by introducing a bent capillary pipette into the plaque center and aspirating gently. The pipette was washed in 0.7 ml of Hanks' salt solution. Aliquots of 0.2 ml of the washing were incubated in each of 3 tubes. To one tube was added Type I polioantibody (monkey antiserum, N.F.), to a second Type III polioantiserum and to a third both Type I and Type III antibodies. The cultures were incubated for 7 days. In this way virus was recovered from 32 of 40 plaques, but in no instance were both types of poliovirus isolated from the same plaque. It is noteworthy that in these experiments poliovirus was recovered from the walls of 2 of the 12 bottles and that the edge of a plaque caused by one type was at times only 2 to 3 mm from that of a plaque caused by the other type of virus.

In the foregoing experiment neutral red was included initially in the overlay. With certain viruses (i.e. measles) it has been found necessary to add the dye several days after inoculation since it interferes with plaque formation. The experiment was therefore repeated in essentially the same way except that neutral red (0.5 ml of 1:2000 solution) was added on the 5th day after inoculation.

Virus isolations were attempted from 30

TABLE II. Recovery of Poliovirus Type I from Glass Walls of Bottle Cultures.

	Bottle A		Bottle B	
Elapse from inoculation	No. of plaques	pfu of virus recovered	No. of plaques	pfu of virus recovered
30 min.	0	0	0	0
1 day	0	0	0	0
2 days	1	0	8	0
3 "	3	$10^{3.1}$	11	0
4 "	3	$10^{3.3}$	11	$10^{1.8}$

plaques about 3 hours after addition of the dye. Polioviruses representing both types were recovered from each of the plaques tested.

Discussion. Lateral migration of virus through agar from the edge of plaques was demonstrated by Dulbecco and Vogt (2). It is evident from results here described that virus also diffuses vertically and can be recovered from the surface over the plaque at the time the plaque is large enough to be visible. These experiments also suggest that this surface virus remains relatively localized in undisturbed bottles, but is distributed over the entire surface of the agar if fluid drains across it.

The most likely source of virus that has been demonstrated on the glass walls of bottles would seem to be drainage between the cell layer and the agar from infected centers. That such flow can occur is suggested by the wedge-shaped extension to the edge of the cell sheet of peripheral plaques which is occasionally observed when incubation is continued several days after maximal count is obtained. It seems less likely that virus on the glass has drained from the agar surface, although this possibility cannot be excluded.

Taken as a whole our experiments suggest that if a single agar overlay is applied and maintained in the inverted position virus isolated from a plaque is very likely to be pure. If this procedure is followed the bottle technique would appear to be acceptable as a method for cloning viruses. Even so, homogeneity of viral progeny cannnot, however, be accepted on a strictly theoretical basis. A requirement for addition of a second agar overlay or fluid such as neutral red after the virus has multipled sufficiently to have diffused through the agar appears to render the procedure unacceptable unless a modification can be found that will prevent widespread dissemination of virus. It would seem that application of a second overlay of agar or of fluid would also be contraindicated when petri dishes were employed according to the procedure of Dulbecco.

Conclusion. The bottle technique cannot be confidently applied to the purification of viruses unless certain precautions are taken to prevent diffusion of virus over the agar surface. These precautions consist in a) maintenance of the system in the inverted position, b) the avoidance of fluids applied to the surface after viral multiplication has taken place.

Summary. Poliovirus was regularly recovered from the agar surface of bottle cultures exhibiting plaques. From the area immediately surrounding a plaque virus recovery was frequent. Only rarely was virus isolated at a distance therefrom. Virus was also often recovered from the glass walls. In cultures simultaneously inoculated with 2 different antigenic types of poliovirus and no material subsequently added, the agent isolated from a plaque consisted of a single antigenic type provided the bottles were maintained in the inverted position. When a solution of neutral red was added several days after inoculation of 2 antigenic types of virus, both types were recovered from a single plaque.

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Effect of Folic Acid on *in vitro* Uptake of Labeled Thymidine by Leukemic Cells.* (26953)

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Long term cultivation of hemic cells *in vitro* is difficult, although de Bruyn (1) reported success with cells from lymphosarcoma and myelosarcoma of mice and Osgood and Brooke (2) established continuous cell lines from human leukemias using gradient culture. Of the virus-induced leukemias, only cells from the fowl leukosis complex have been cultivated. Beaudreau *et al.* (3) have successfully grown myeloblastosis, and Lagerlof (4), erythroleukemia. Although it is possible to propagate at least 2 mouse leukemia viruses in mouse embryo cells, Friend's (5) and Moloney's (6), the cells themselves do not proliferate continuously.

Fischer's observations that the ascitic form of the mouse leukemia L5178 could be grown *in vitro* only in the presence of high concentrations of folic acid stimulated interest in determining the relationship of this compound to leukemias (7,8). L5178 cells are deficient in the enzyme which converts dihydrofolic acid to tetrahydrofolic acid (7). In addition, in human leukemia the folic acid content in white cells is higher than in the normal cells (9,10).

Because of the implication that folic acid is intimately involved in the leukemic process, it was decided to study the synthesis of deoxyribonucleic acid (DNA) in different virus-induced and non-virus-induced leukemias and lymphosarcomas growing in culture in presence of increased amounts of folic acid. This was done by adding labeled thymidine (H^3) to cultures and determining the number of labeled cells using radioautographic techniques (11). Mitotic indices were determined on the same specimens.

Materials and methods. Cultures. To obtain a monolayer of cells, the explant was placed on a coverslip in a very thin plasma clot. Preparations were made from spleens of mice infected with Friend's leukemia§ (12), Gross's leukemia (Passage A)§ (13), Moloney's leukemia§ (14), and L1210‡. A mouse lymphosarcoma isolated from random-bred stock mice and kept in serial passage was also used. Normal splenic cultures served as controls. The cultures were divided into 2 groups, one grown in medium A consisting of Eagle's basic medium (15) with 15% inactivated horse serum and 1 mg/liter of folic acid (15,16); the other grown in medium B, identical with medium A except that it contained 10 mg/liter of folic acid.

Radioautographs. After 10-12 days culture the explants had grown out as monolayers and the plasma clots were completely digested. Tritiated thymidine (specific activity 3 curie/mM) was added to the medium to give a final concentration of 0.1 μc/ml. After 3-48 hours, cultures were sacrificed and the number of labeled cells determined. The cultures were washed in distilled water and were fixed in methyl alcohol-acetic acid (3:1). They were washed again in distilled water and allowed to dry. The stripping film technique described by Fitzgerald *et al.* (17) was used; the film was Kodak AR-10. After developing, slides were stained with Giemsa at pH 7. It was found that after 24 hours half of the cells

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§ Received through the courtesy of the respective authors.

‡ L1210 was induced by carcinogenic agents by Dr. L. W. Law and was obtained from Dr. Joseph Burchenal of this Institute.

TABLE I. Incorporation of Tritiated Thymidine by Various Tissues and % Mitosis.

Tissue	Media*	% labeled cells	% mitosis
Friend's leukemia spleen	A	25 ± 4	.9 ± .1
	B	49 ± 4	1.4 ± .1
Gross's leukemia spleen	A	26 ± 1	.7 ± .1
	B	43 ± 1	1.5 ± .1
Moloney's leukemia spleen	A	25 ± 2	.7 ± .1
	B	42 ± 1	1.3 ± .2
L1210 leukemia spleen	A	28 ± 2	.7 ± .2
	B	29 ± 3	.9 ± .1
Lymphosarcoma	A	31 ± 3	1.1 ± .3
	B	25 ± 2	1.0 ± .3
Normal spleen	A	42 ± 1	1.5 ± .1
	B	44 ± 2	1.4 ± .1

* Eagle's media containing 1 mg/l (A) or 10 mg/l (B) folic acid.

were labeled, so this time was chosen for the experiments.

Cell counts. One thousand cells per culture were counted using the oil immersion objective, and the number of cells containing grains in the nucleus was determined. The number of grains in the nucleus varied considerably from a few to many, but this was disregarded, since the same variability was noted in all groups. The results are reported as the per cent of labeled nuclei in the total number of nuclei counted. The number of mitoses per 100 cells was also recorded.

Results. Growth in tissue culture. The number and types of cells which grow out from splenic cultures, whether malignant or normal, are of many different types whose exact origin is unknown. In this study they were classified mainly as 2 types, a fibroblast-like cell and a round basophilic cell. In the L1210, Gross's and Moloney's leukemia, and lymphosacroma, the latter type predominated; in cultures of the Friend leukemic spleen most cells were also basophilic but larger than the others.

Incorporation of tritiated thymidine and per cent mitosis. The results are shown in Table I. The uptake of tritiated thymidine was more pronounced in normal spleen than in any of the leukemic spleens or in the spleen from the mouse having lymphosarcoma. These results agreed with those of others (18,19). In the presence of 10-fold folic acid (medium B), the virus leukemic spleens took up significantly more labeled thymidine than did the L1210 or the lymphosarcoma. This appeared to be a reflection of increased growth, since in most instances the mitotic index almost doubled.

The Friend leukemia agent was cultivated in Scherer's medium (20) which contains purines and pyrimidines available for nucleic acid synthesis. In this medium, 29% of the cells were labeled. When ten times the amount of folic acid was added, however, the number of labeled cells was 40%. Thus, the stimulatory effect was apparent.

To determine if uptake of thymidine was related to DNA synthesis and mitosis, a correlation analysis was done by the method of Snedecor (21). Fig. 1 shows the adjusted values and the real values. The low values represent the L1210 and lymphosarcoma in both media and the 3 virus leukemias in medium A. The higher values represent the normal spleen (both media)

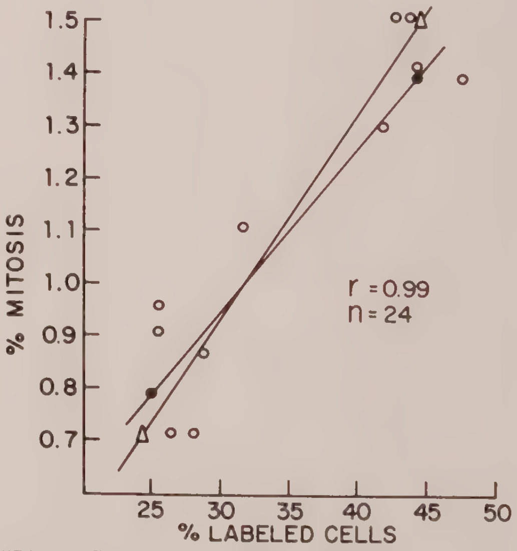

FIG. 1. Correlation analysis showing the relationship of uptake of tritiated thymidine to DNA synthesis and growth: adjusted values of per cent mitosis (●); adjusted values of per cent labeled cells (△); real values (○).

TABLE II. Incorporation of Tritiated Thymidine into Normal Splenic Cells Infected with Friend's Leukemia Virus.

Type of tissue	Media*	% labeled cells
Normal spleen infected with virus	A	17 ± 3
	B	31 ± 5
Normal spleen, control	A	40 ± 2
	B	44 ± 4

* Eagle's media containing 1 mg/l (A) or 10 mg/l (B) folic acid.

and the 3 virus leukemic spleens in the media with the increased amounts of folic acid. The correlation coefficient shows that uptake of tritiated thymidine is related to DNA synthesis and growth as measured by the mitotic index.

To see if there were some relation between the folic acid effect and the presence of virus, another type of experiment was set up. Normal spleen was grown out in tissue culture and, after 8 days, infected with the Friend leukemia virus. Radioactive thymidine was added 3 days later. It can be seen (Table II) that the normal spleen infected with virus had a much lower per cent of labeled cells than did the control uninfected spleen grown in the same medium. However, when the infected spleen was grown in medium with excess folic acid, the per cent of labeled cells almost doubled, although the number of labeled cells was not as great as in the normal control cultures.

Discussion. The tissue culture experiments reported here agree with the findings of Gavosto *et al.* (19) and Craddock (18) who showed that in short-term experiments the uptake of tritiated thymidine is less in human acute leukemic cells than in normal white cells. It was also demonstrated that certain leukemic spleens need a supplement of folic acid in order to get values equal to that of normal spleen.

Folic acid serves as a coenzyme in utilization of one carbon compounds (22) which serve as the building blocks in biosynthesis of purines, pyrimidines, and the amino acids, serine and methionine. It is required for growth of cells in tissue culture at a level of 1 mg/liter, as shown by Eagle (16). This amount proved to be adequate for the growth of normal mouse spleen, but the 3 virus-infected spleens studied required 10 times that amount. This was true even in the presence of purines and pyrimidines in the medium. The fact that these are virus-induced leukemias and that similar results were obtained with normal spleens infected with virus suggests that perhaps this is a phenomenon associated with viral infection. However, it has been reported (7,8,23) that two types of mouse leukemias (L5178 and P815), supposedly not of viral origin, have increased requirements for folic acid; the need for folic acid may be a matter of individual cellular requirements rather than of the etiologic agent. The difference shown may be more complex than it appears, since attempts to grow the Friend leukemia virus in continuous passage with increased amounts of folic acid have not enhanced the production of virus.

Summary. The uptake of tritiated thymidine by leukemic and normal spleens has been studied. The values obtained for the normal spleen were always higher than those for the leukemic spleen. When virus-induced leukemic spleens were cultured in high concentrations of folic acid their uptake equalled that of normal spleen. This was not true of a non-viral leukemia or of a lymphosarcoma. When normal splenic cultures were infected with the Friend leukemia virus, the number of labeled cells decreased; but when high concentrations of folic acid were added, the number increased.

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Efficiency of Plaquing Certain Poliovirus on Tissue Cultures at Higher Temperatures as a Function of L-Cystine Concentration.* (26954)

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In 1956 3 wild-type polioviruses were shown to require exogenously supplied cystine for efficient plaque production on monkey kidney tissue cultures at 36° (1). Subsequently, it was shown that their efficiency of plaque production on cultures not supplied with cystine is dependent on the temperature during plaque development: lowering the temperature below 37° increased this efficiency (2).

That the temperature of the host cell has a marked influence on growth of poliovirus has recently been well recognized [see reviews by Lwoff and Lwoff (3) and by Sabin (4)]. However, the relation of cystine to this influence of temperature has not been adequately explored; the results of such investigation are described here.

Materials and methods. Polioviruses. The wild-type virus, the 30°-adapted (ca^{30}) mutant (5), and the 23°-adapted (ca^{23}) mutant (6) of the Akron strain of antigenic type 1 poliovirus were used. *Growth of monkey kidney tissue cultures (MKTC's).* Kidney cells from cynomolgus monkeys (*Macaca irus*) or rhesus monkeys (*M. mulatta*) were dispersed with trypsin (7), suspended in lactalbumin growth medium (8), delivered to Petri plates, and then kept at 37°-38° in an atmosphere of 3% CO_2 and 97% air for 5-9 days, after which time suitable sheets of cells had developed. *Inoculations and plaque development.* The sheets were washed twice, each time with 4 ml phosphate-buffered saline (PBS) (9), then were inoculated with the appropriate virus concentrations, 0.3 ml inoculum per plate. Inoculated sheets were put at 37° for 30 minutes. Then a sheet received 6 ml altered Eagle's maintenance medium (AE_m) (2), adjusted with HCl and/or NaOH usually to pH 6.9-7.1, containing 0.9% washed (9) agar and 0, 0.025, 0.050, 0.20, or 1.0 mM

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L-cystine: during plaque development the pH was somewhat higher, ranging from about 7.2 to about 7.7. In some experiments the effect of purposely varying the pH during plaque development was assessed; for these the initial pH of the agar-AE$_m$ was adjusted with HCl and/or NaOH to various values between 5.7 and 7.8. The pH of the agar-overlay during plaque development was measured periodically either colorimetrically, using phenol red as pH-indicator and standard plates containing phosphate buffers of known pH's, or with a pH meter. For some tests (Table II) compounds not present in this medium were added; or concentration of DL-methionine was increased. The plates were then put at various temperatures (29°-41°) in an atmosphere of 3% CO_2 and 97% air. Plates were kept under these conditions 4 days (wild-type and ca^{30} mutant) or 5 days (ca^{23} mutant); the temperature was measured periodically and the mean calculated. The surviving cells were stained with neutral red. *Calculation of efficiency of plating (eop.).* Within certain temperature and L-cystine concentration ranges the titer of a given virus population was high and independent of the temperature and L-cystine concentration. Such high titers, which are maximal for the given virus populations in this MKTC system, were used as standards. The titer of a population under other conditions, e.g. at a higher temperature, was divided by its maximal titer to give its eop under these other conditions; an eop so calculated is of course a relative one.

Results. Akron plaque production as a function of temperature and the supply of L-cystine. In Table I are presented data from a representative experiment showing the promotion by L-cystine of plaque development by the Akron ca^{30} mutant on MKTC cells at a relatively high temperature. Curves which show growth of poliovirus on cells *in vitro* as a function of temperature are typically steep at the warmer end; i.e., when the temperature exceeds a certain value, growth of the virus decreases rapidly with increasing temperature (10, 11, 12). By graphing the data from Table I and other experiments we see (Fig. 1) that the position of this steep portion is displaced toward higher temperatures by increasing the concentration of L-cystine in the medium over the cells. This displacement was observed with all 3 Akron viruses, which represent 3 degrees of genetic ability to grow at relatively high temperatures: with the initial concentration of L-cystine in the medium at the rather low value of 0.025 mM, the steep portion commences at about 30.7°, 36.2°, and 37.4° for the ca^{23}, ca^{30}, and wild-type viruses, respectively (see curves for the 2 mutants in Fig. 1). During

TABLE I. Promotion by L-cystine of Development of Plaques of the ca^{30} Mutant at 37.3°.

Conc. (mM) of L-cystine	No. plaques/plate when inoc. virus conc. is				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
0	ov	16	0	*	*
.025	*	80	3	1	*
.050	*	*	46	11	0
1.0	*	*	*	ov	30†

ov = extensive overlap of plaques.
* Not done. † eop = 1.

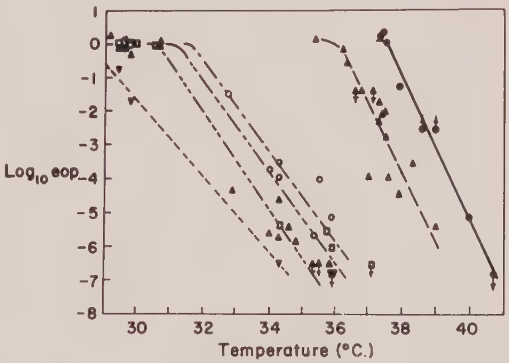

FIG. 1. Plaque production by ca^{30} and ca^{23} mutants as a function of temperature and supply of L-cystine. The 4 curves at left (open symbols) are for the ca^{23} mutant and the 2 at right (closed symbols) for the ca^{30} mutant. Results with media containing 0, 0.025, 0.20, and 1.0 mM L-cystine are indicated by triangles with horizontal side up, triangles with horizontal side down, rectangles, and circles, respectively. Points with arrows pointing up and down indicate minimal and maximal values, respectively.

TABLE II. Capacities of Several Compounds to Enable the ca^{23} Mutant to Grow at 34°.

Added compound*	No. of plaques per plate†			
	Exp. T192		Exp. T202	
	Individual plates	Arithmetic mean	Individual plates	Arithmetic mean
None	9; 5; 7	7.0	0; 2; 0	.7
DL-cystathionine	25; 11; 26	20.7	12; 0; 1; 0	3.2
L-cysteine	90; 86; 100	92.0	24; 45; 35	34.7
D-cystine	19; 6; 15	13.3	12; 36; 2	16.7
L-cystine	100; 80; 110	96.7	36; 58; 80; 68	60.5
S-ethyl L-cysteine	7; 16; 4	9.0	2; 0; 2	1.3
Glutathione	‡		14; 58; 16	29.3
DL-homocysteine thiolactone	3; 1; 5	3.0	3; 0; 3	2.0
2-mercaptoacetic acid	‡		4; 0; 3	2.3
DL-methionine	‡		0; 2; 0	.7
DL-serine	‡		1; 0; 2	1.0
Sodium sulfate	5; 2; 4	3.7	0; 1; 0	.3

* Basal medium was AE_m containing 0.025 mM L-cystine. This medium contains 0.10 mM DL-methionine but none of the other 9 compounds listed above. Where additional L-cystine or DL-methionine was added, the former was present at 1.0 mM and the latter at 2.05 mM. Where added, D-cystine was at 0.975 mM and each of the other 8 compounds was at 1.95 mM.

† Each plate was inoculated with 340,000 plaque-forming units, based on assay of the viral populations at 30°. The rather large intragroup interplate variation in number of plaques/plate is probably mainly due to variation in temperature from point to point within the continuous-flow incubator space used for the experimental plates: for example, a difference of only 0.3° will result in a 2- to 3-fold difference in number of plaques produced (Fig. 1).

‡ Not done.

plaque development the pH ranged from about 7.2 to about 7.7 in these experiments. In a given experiment the pH of the medium with 1.0 mM L-cystine was usually about 0.1-0.2 lower than that of the media with low concentrations, e.g. 0.025 mM, of L-cystine. A similar, or greater, lowering of the pH of a low-cystine medium during plaque development simply by adjusting its initial pH to values <6.9 did not increase the eop on warm cells†: thus L-cystine does not enable plaque development on warm cells simply by lowering the pH slightly. Conversely, increasing the pH of a high-cystine medium during development by adjusting the initial pH to values somewhat >7.1 did not decrease the eop on warm cells. Thus L-cystine enables these Akron viruses to grow on relatively warm cells over a pH range at least from 7.1 to 8.1.

Substitutes for L-cystine. Ten compounds were compared with L-cystine in ability to enable Akron ca^{23} plaque development at 34° (Table II). High activity was shown by L-cysteine and glutathione, and slight activity by DL-cystathionine [cf. (1)] and D-cystine.‡

Genetic revertants. Viral progeny isolated from plaques produced by the ca^{23} and ca^{30} viruses under conditions where eop was very low because temperature was too high for concentration of L-cystine used were found to be genetically reverted. The increased capacity of such revertant populations to grow at comparatively high temperatures is shown in Table III. For comparison, in this table are also shown 2 nonrevertant progeny populations (isolates 3 and 4 from ca^{30} virus), obtained from plaques produced under conditions (at 37° but with 1.0 mM L-cystine) giving high eop.

Discussion. Poliovirus growth as a function of the interrelation of the genetic na-

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† When the pH of the agar-medium during plaque development fell below about 6.8, the efficiency of plating of the ca^{30} mutant dropped sharply, with or without a high supply of L-cystine (cf. 13).

‡ The possibility that the low activity of our sample of D-cystine was due to contaminating L-cystine should be mentioned.

TABLE III. Tests of Ability of Progenies from Akron ca^{23} and ca^{30} Viruses to Grow at Comparatively High Temperatures.

Akron virus plated	Plaque development: Conc. (mM) of L-cystine	Temp.	Log$_{10}$ eop	Plaque isolate No.	Test exp.*	Isolate's log$_{10}$ eop	Parent's log$_{10}$ eop in same test†
ca^{23}	.025	34.6°	–5.55	1	A	.62	–5.86
				2		1.09	
	1.0	34.3°	–3.96	3	B	.26	–5.56
				4		– .48	
ca^{30}	.025	39.0°	–5.47	1	C	.88	–3.47
				2		.43	
	1.0	37.0°	– .44	3	D	– .97	–1.63
				4		–1.74	

* Medium contained 0.025 mM L-cystine. Lower temperatures were 30.7°, 29.7°, 29.5°, and 29.6° and higher temperatures 34.8°, 34.6°, 38.3°, and 37.3° for test experiments A, B, C, and D, respectively.

† Included for comparison with preceding column.

ture of virus, temperature, and concentration of L-cystine. L-cystine enables an Akron poliovirus to grow at relatively high temperatures. Conversely, lowering the temperature enables the virus to grow on MKTC cells in a medium with a relatively low concentration of L-cystine. For MKTC's at a given temperature and on a medium with a given concentration of L-cystine, growth of poliovirus is dependent on the genetic nature of the inoculated virus. Thus it is clear that poliovirus plaque production of MKTC's is dependent on the interrelations of these 3 variables. From this it appears that the specification of the temperature above which a given poliovirus plates inefficiently, i.e. eop is $< 10^{-2}$ or some other arbitrary fraction, should also specify the concentration of L-cystine used. Whether L-cystine enables a poliovirus to grow at relatively high temperatures on monkey cells *in vivo* is not known. If it does, then it probably acts similarly in man; and we might expect the course of poliomyelitis to be dependent not only on such other things as rate of appearance of antibodies but also on the interrelation among the individual's fever response, his L-cystine nutrition, and the genetic capacity of the infecting poliovirus to grow at higher temperatures. These considerations further suggest that topical administration, e.g. in the skin, which is relatively cool, of L-cystine with a poliovirus of low genetic capacity to grow at higher temperatures might enable local viral growth sufficient to stimulate antibody production.

Viral mutation leading to elevation of maximal temperature at which a poliovirus grows efficiently. The relatively hot-adapted revertant polioviruses arose by mutations which probably occurred during the very limited growth of the parental ca^{23} and ca^{30} viruses at the high temperatures. At high temperatures where the eop was very low L-cystine increased the number of such revertant-containing plaques apparently simply by increasing this limited parental growth and thus the opportunties for mutation. Under conditions where the eop approached 10° due to the high concentration of L-cystine the plaques contained virus like the parent, as expected since under these conditions the selective advantage of hot-adapted revertants was small or zero. These hot-adapted mutants are very probably essentially similar to the hot-adapted polioviruses, derived from wild-type, obtained by Lwoff and Lwoff (18) through serial passage at comparatively high temperatures.

An hypothesis regarding how L-cystine enables poliovirus to grow at higher temperatures. As released from the cell, poliovirus is in a thermolabile state: its *in vitro* stability is low at 45°-53°. Such thermolabile particles can be greatly stabilized against thermal inactivation by *in vitro* reaction with L-cystine (14, 15): apparently the protein coat of a thermolabile particle reacts with several molecules of L-cystine, resulting in a coat much less easily denatured by heat (14). Applying this idea to the effect of L-cystine on viral growth in cells at different temperatures, we hypothesize that L-cystine stabilizes precursor viral protein against thermal denaturation at 31°-41°. Such an hypothesis appears compatible with (a) the finding of Lwoff and Lwoff (16) that a relatively late stage of intracellular viral development is inhibited by high temperature, (b) the report by Darnell and Levintow (17) that poliovirus protein synthesis proceeds rapidly at a similarly late stage of intracellular viral development, and (c) the very high dependence of rate of protein denaturation on temperature.

Summary. L-Cystine enables viruses of the Akron strain of antigenic type 1 poliovirus to grow on *in vitro* monkey kidney cells at comparatively high temperatures; at lower temperatures little or no exogenously supplied L-cystine is needed by these viruses. These results were shown for Akron viruses of 3 different degrees of genetic ability to grow at higher temperatures. The activity of L-cystine is not due to its slight lowering of the pH during plaque development. L-Cysteine and glutathione are active, DL-cystathionine is slightly active, and a sample of D-cystine showed slight activity. Rare plaques produced at temperatures too high for the L-cystine supply contained hot-adapted mutants.

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A Comparison of Canine Prostate and Wheat Germ Oil Acid Phosphatase.* (26955)

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The acid phosphatase of the canine prostatic fluid is a relatively stable enzyme which is little affected in the course of a week by significant concentrations of proteolytic enzymes present in the same fluid (1, 2). Evidence exists that a number of acid phosphatases occur in nature (3-8). In this study the acid phosphatase of canine prostatic fluid is compared with that of wheat germ oil for its action on natural and synthetic substrates at different pH levels.

Methods. Methods developed primarily for estimation of serum alkaline phosphatase (9) have been extended to the study of acid phosphatase simply through the employment of a buffer system with a lower pH. In the present study, monophenylphosphate (9), β-glycerophosphate (10), phenolphthalien diphosphoric acid (11), glucose-1-phosphate and o-carboxyphenylphosphate (12), were used for a comparative study at various pH levels between 4 and 7.

The details of the methods used are as follows: (1) *β-glycerophosphate*—4.5 ml of 1% sodium β-glycerophosphate buffer were incubated with 0.1 ml canine prostatic fluid for 1 hour at 37.5° C in an atmosphere of air. The incubation was carried out in 12 ml heavy-walled centrifuge tubes so that enzyme inactivation with 1 ml 30% trichloroacetic acid and centrifugation would not require further transfers. Control tubes were incubated without prostatic fluid, the latter being introduced after the protein precipitant. One ml of the supernatant was mixed with 1 ml molybdic acid and 0.4 ml of the Fiske-SubbaRow reagent. The solution was diluted to 9 ml with water and read at 660 mμ.

(2) *Phenolphthalien diphosphoric acid*—0.3 ml prostatic fluid and 1 ml 0.06 M potassium acid phthalate containing 0.72 mg phenolphthalien diphosphoric acid were permitted to stand at room temperature for 2 minutes. The reaction was stopped with addition of 8.5 ml of 0.2 M carbonate-bicarbonate buffer (pH 10.4) and the dephosphorylated phenolphthalien was measured at 550 mμ. When the release of phosphate was followed, 1 ml of 10% trichloroacetic acid was substituted for the carbonate-bicarbonate buffer.

(3) *o-Carboxyphenylphosphate*—To 2 ml of 0.15 M acetate—HCl buffer were added 0.1 ml prostatic fluid and 0.5 ml of 0.00365 M o-carboxyphenylphosphate. The reaction was followed by recording absorption at 298 mμ. every 2 min. The readings at 20 min. were used for calculations. When the release of phosphate was followed, trichloroacetic acid was used as above.

(4) *Monophenylphosphate*—2.5 ml of 0.19% monophenylphosphate were combined with 2.5 ml of a 0.2 M Tris-acetate buffer to which 0.1 ml prostatic fluid was added. The solution was incubated for 30 min. at 37.5° C and the reaction halted with the addition of 1 ml 30% trichloroacetic acid. Three ml of the supernatant were transferred to a cuvette and optical density obtained at 270 mμ. An aliquot was taken for phosphate determination by the Fiske-SubbaRow procedure.

The tris-acetate system was also utilized for comparing β-glycerophosphate with glucose-1-phosphate. Two-tenths ml of 0.064 M substrate was used and release of inorganic phosphate was determined as above.

The method for collecting and preparing the canine prostatic fluid is described elsewhere (1). Wheat germ acid phosphatase

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TABLE I. Comparison of Activities of Canine Prostatic and Wheat Germ Acid Phosphatase Using Release of Phosphate as End Point.

Substrate	Source of acid phosphatase	Amt of enzyme or fluid	Buffer system	mg PO_4 = released/hr/ml fluid			
				pH 4	5	6	7
β-glycerophosphate	Wheat germ	.1 mg	Glycerophosphate	.43	.54	.40	.11
"	" "	2.0 "	"		14.3	10.6	5.5
"	Prostatic fluid 353*	.1 ml	"	2.1	2.6	3.4	2.5
"	" " 362	"	"	5.3	7.0	8.2	5.6
"	" " 368	"	"	12.1	13.1	14.8	11.9
"	" " 379	"	"	8.1	9.3	11.1	7.9
"	" " 385	"	"	5.3	6.8	8.4	5.8
β-glycerophosphate	Wheat germ	2.0 mg	Tris-acetate		12.6	7.5	4.3
"	Prostatic fluid 387	.1 ml	"	3.0	3.5	3.2	2.0
"	" " 386	"	"	2.9	3.7	3.4	2.4
Glucose-1-phosphate	Wheat germ	2.0 mg	"		6.0	4.4	1.7
"	Prostatic fluid 368	.1 ml	"	3.2	3.1	3.6	2.4
"	" " 386	"	"	1.9	2.1	1.7	1.1
"	" " 379	"	"	1.9	2.6	1.9	1.6
"	" " 385	"	"	1.3	3.2	1.5	.9
Phenylphosphate	Wheat germ	.5 mg	"	3.8	6.4	5.8	2.3
"	Prostatic fluid dog pool	.1 ml	"	6.6	7.8	6.8	4.9

* Number assigned to each dog.

was obtained from Worthington Biochemical Corp.

Results and discussion. Data in Table I indicate that whereas wheat germ acid phosphatase optimally releases phosphate from β-glycerophosphate near pH 5, prostatic acid phosphatase functions more actively near pH 6. However, in a tris-acetate buffer both enzymes operate maximally near pH 5. This is true for the natural substrates, glucose-1-phosphate and β-glycerophosphate as well as synthetic organic phosphate, monophenylphosphate.

A decreased release of inorganic phosphate from glucose-1-phosphate was observed with both enzymes indicating a lower affinity of this substrate for acid phosphatase whereas using synthetic substrates both enzymes showed optimum activity at pH 5 (Table II). With prostatic fluid en-

TABLE II. Comparison of Activities of Canine Prostatic and Wheat Germ Acid Phosphatase Using Release of Phenyl Group as End Point.

Substrate	Source of acid phosphatase	Amt of enzyme or fluid	Buffer system	mg phenyl group released per unit time per ml fluid*			
				pH 4	5	6	7
Monophenylphosphate	Wheat germ	.5 mg	Tris-acetate	1.52	2.78	2.25	.75
"	Prostatic fluid 368†	.1 ml	"	3.35	3.63	3.0	2.6
"	" " 369	"	"	2.79	3.10	2.76	1.96
o-carboxyphenylphosphate	Wheat germ	.1 mg	Acetate-HCl	.02	.03	.01	.006
"	Prostatic fluid 353	.1 ml	"	.22	.58	.54	.41
"	" " 385	"	"	.19	.43	.40	.31
Phenophthalien	Wheat germ	.6 mg	Potassium acid	.012	.022	.016	.004
Diphosphoric acid	Prostatic fluid 368	.3 ml	Phthlate	.053	.085	.099	.081
" "	Prostatic fluid pooled sample	"	"	.024	.034	.046	.033

* Time indicated in procedures.

† Number assigned to dog.

TABLE III. Stoichiometry in Release of Phenyl and Phosphate Groups by Acid Phosphatase from Synthetic Organic Phosphates.*

Substrate	pH	Phenyl group released		Phosphate group released	
		Wheat germ	Prostatic fluid	Wheat germ	Prostatic fluid
		μM			
Monophenylphosphate	4	.27	.50	.32	.56
	5	.48	.55	.55	.65
	6	.40	.49	.50	.58
	7	.13	.35	.20	.42
o-carboxylphenylphosphate	4	.08	.29	.07	.41
	5	.09	.35	.10	.44
	6	.06	.31	.04	.31
	7	.05	.24	.02	.29
Phenolphthalien diphosphoric acid	4	.04	.08	.09	.17
	5	.07	.11	.13	.24
	6	.05	.15	.13	.29
	7	.02	.11	.11	.23

* Concentrations of organic phosphates used were those recommended by the individual procedures; 0.5 mg wheat germ and 0.1 ml canine prostatic fluid acid phosphatases, respectively, were compared.

zyme phenolphthalien diphosphoric acid provided an exception with an optimum at pH 6.

The data in Table III disclose the stoichiometry of the release of phosphate and phenyl groups in each method investigated here. In the case of phenolphthalien diphosphoric acid approximately 2 molecules of phosphate were obtained for each molecule of phenolphthalien.

Summary. Canine prostatic fluid contained a potent acid phosphatase which was more active against the natural β-glycerophosphate than several synthetic organic phosphates tested. A distinction between the enzymes from prostatic fluid and wheat germ oil could be made on the basis of optimal pH requirement. Stoichiometric determinations indicated that the simplicity of phosphate determination was to be preferred to measurement of the released phenyl group derivative.

We wish to express our gratitude to Dr. Marcus M. Mason for his continued encouragement in these investigations.

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Observations on the Relationship Between Hemagglutinin and Neuraminidase of Influenza Viruses.* (26956)

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Evidence has been presented (1) suggesting that hemagglutinin and neuraminidase may represent 2 distinct and independently functioning attributes of the myxovirus particle, a possibility also suggested by other workers (2). In substantiation of this hypothesis, it was shown that certain strains of influenza virus could be freed of enzyme without significant loss of hemagglutinin (i.e. converted to indicator virus) while others could be stripped of hemagglutinin without significant loss of neuraminidase activity. Moreover the presence or absence of detectable neuraminidase activity in formalinized virus appeared to bear little relation to the enhanced susceptibility of the hemagglutinin in these preparations to inhibition by mucoids over that of the corresponding infective viruses. The relation between the susceptibility of certain mucoids to viral enzyme and the capacity of these substrates to inhibit viral hemagglutination has also been investigated. A lack of correlation has been found which further substantiates the dissociation of hemagglutinin and enzyme. The results of these studies form the subject of this report.

Materials and methods. Viruses were propagated and titrated, and hemagglutination inhibition tests performed as previously described (3,4). Strains of Asian influenza virus (RI/5+ and RI/5—) were generously supplied by Dr. P. W. Choppin and a strain of Newcastle disease virus (NDV) by Dr. P. I. Marcus. Sputum inhibitor was prepared by the acid precipitation method of Marmion, Curtain and Pye (5) as previously reported (6). Urinary mucoprotein was prepared according to the procedure of Tamm and Horsfall (7) from urine of the same donor, a secretor of blood group B. Brain mucolipid was kindly supplied by Drs. A. Rosenberg and E. Chargaff (8); bovine submaxillary mucoid by Drs. R. Heimer and K. Meyer (9); and serum orosomucoid by Dr. S. A. Ellison. While preparing normal human amnion cell cultures for purposes unrelated to the present problem, it had been noted that in the process of trypsinization large amounts of mucoid material were liberated into the suspending medium as the amnionic tissue became disaggregated. After removal of the cells by centrifugation, the supernatants were dialyzed, clarified by centrifugation and lyophilized. The resulting completely soluble colorless material, doubtless largely protein in nature, was found to contain appreciable sialic acid. Amnionic mucoid from placentas of blood group A_1 (cord blood) was devoid of blood group A activity. One preparation from a placenta of group O inhibited agglutination of group O erythrocytes by *Ulex Europaeus* extract but not that by bovine anti-H serum absorbed with A_1B erythrocytes. Receptor substance from human, guinea pig, and sheep erythrocytes was obtained as previously described (3). In addition, the procedure described by Kathan, et al. (10) was employed. This is based primarily on the method originally introduced by Westphal et al. (11) for dissociation of lipoprotein-carbohydrate complexes with hot phenol. As starting material, a lipid-rich (LR) fraction (12) of human erythrocyte stroma was employed. The final product in each

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instance was comparable in all respects to a sample of receptor substance generously supplied by Dr. R. H. Kathan (prep. 1, Table II). Sialic acid was measured by the Bial orcinol and direct Ehrlich reactions (13) as well as by the thiobarbituric acid method (14), using crystalline N-acetylneuraminic acid† (NANA) from sheep serum as primary standard. Chromatographic identification of NANA was carried out as previously described (1).

Results and discussion. It will be seen from Table I that sputum mucoid was relatively active against 4 strains (PR301, FM1, Lee and GL) while the activity of brain mucolipid, both of human and bovine origin, was limited to PR8 and PR301, and that of bovine submaxillary mucoid to PR8, Lee and NWS. The latter finding is in agreement with the observations of other investigators (15,16) who have found bovine submaxillary mucoid to be restricted in inhibitory range among numerous strains of influenza virus. Amnionic mucoid was devoid of detectable inhibitory activity; but there is no way of being certain whether or not receptors may have been present initially in the intact membranes and subsequently destroyed by the trypsinization procedure with which this material was isolated. None of the amnionic mucoid preparations contained hemagglutinin in the concentrations used, although there were traces of residual tryptic activity. The inhibitor of normal horse serum was active against 2 strains of Asian influenza virus (strain RI/5+ of Choppin and Tamm, and our strain of A2/Japan/305/57) and against FM1. The sharp differences noted in the range of inhibitory activity against representative strains of myxovirus is doubtless a reflection of basic structural dissimilarities of the active components in the various mucoids as suggested by the work of Gottschalk and de St Groth (16). Collocalia mucoid inhibited all strains of myxovirus hemagglutinin including RI/5−, a finding which suggests that this material contains inhibitory components resembling those of horse serum.

The susceptibility of the mucoids to the action of viral neuraminidase was determined using virus concentrated by ultracentrifugation of freshly harvested infective chorioallantoic fluid. Substrate and virus were mixed in the proportion mg substrate/μg "viral" nitrogen averaging 0.1 (range 0.02-0.25). It will be seen from Table I that treatment of any of the mucoids with one or more strains of active virus resulted in the release of appreciable amounts of free or dialyzable N-acetylneuraminic acid. In a few instances (e.g. bovine submaxillary mucoid with PR301, GL and NWS) the quantities of NANA released, albeit small, were readily identified. The limited enzymatic action noted in these instances may perhaps be explained by relatively unfavorable experimental conditions. For example, quantitative study of the enzymatic action of myxoviruses with Collocalia mucoid has revealed the optimum pH for most strains to be 5.5-6.0 (4), there being a sharp drop in activity above pH 7.0. The earlier experiments reported herein, however, had been carried out at 7.2 in calcium borate buffered saline in accord with the methods previously reported by others (17,18) in which receptor destroying activity rather than quantitative measurement of neuraminidase had been the object of study. It might be noted in this connection that the myxovirus neuraminidase is fully active in the absence of calcium ions. Amnionic mucoid, showing no significant inhibition of hemagglutination, was nonetheless susceptible to viral enzyme, resembling in this respect orosomucoid which, while not capable of inhibiting hemagglutination, has also been found susceptible to myxovirus neuraminidase. There appears then to be little relation between the potency of a given mucoid as an inhibitor of hemagglutination

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† Kindly supplied by Dr. A. Rosenberg.

TABLE I. Hemagglutination Inhibition in Relation to Enzymatic Activity of Myxoviruses.

	Inhibitor (Substrate)*													
	Sputum mucoid		Bovine submaxillary mucoid		Brain mucolipid				Human amnion mucoid§		Horse serum		Collocalia mucoid	
					Human		Bovine		Prep. 1	Prep. 2				
NANA content	4%†		27%†		16%†		20%†		3%‡	3%‡	383 μg/ml‡		16%,† 12%‡	
Virus	IR	% NANA released	IR	% NANA released	IR	% NANA released	IR	% NANA released	% NANA released		IR	% NANA released	IR	% NANA released
PR8	9	60	1	22	4	34	1	25			>1200		2	53
PR301	3	36	>85	4	1	19	1				>1200		2	43
FM1	2	26	>89		>40		> 7		65	61	2		1	52
LEE	2	57	1	21	>81	13	>34				>1200		10	50
GL	1	33	>89	7	>40	+‖	> 7				>1200		10	80
A2/Jap	>17	53	> 8		>34		>23				1	4	4	56
Asian RI/5–								78	80	83	>1200	5	10	55
Asian RI/5+											1	3	1	78
Swine											>1200		2	58
NWS	> 6	36	1	2	>44	11	>40						2	63

* IR = Inhibition ratio: Ratio of μg inhibiting 5 HA units/μg inhibiting 5 HA units of the most sensitive strain(s) taken as 1. Enzyme activity expressed as % of total hydrolyzable chromogen liberated from substrate. Avg ratio: mg substrate/μg ''viral'' nitrogen = 0.1 (range 0.02-0.25).

† Bial orcinol reaction.

‡ Thiobarbituric acid reaction.

§ No significant hemagglutination inhibition with either preparation.

‖ Chromatographic identification only.

and its susceptibility to the enzymatic action of a given strain of myxovirus.

With only one exception, active virus eliminated all detectable hemagglutinin receptors in mucoids normally active against one or more strains of virus. Normal horse serum, however, treated either with concentrated A2/Jap, Asian RI/5+ or RI/5− virus retained most of its inhibitory titer against the first two of these strains. Retention of inhibitory activity following treatment with active virus was also noted by Choppin and Tamm (19). In agreement with their findings also is our observation that all 3 Asian strains showed potent neuraminidase activity against several other mucoids as well, despite the sharp contrast in susceptibility to horse serum inhibition

TABLE II. Interaction of Erythrocyte Stroma Components with Myxovirus Hemagglutinin and Neuraminidase.

Preparation	NANA content‡	PR8	μg inhibiting 5 hemagglutinin units*/(NANA released by active virus†) PR301	FM1	Lee§	GL	A2/Jap	Swine	NWS
Human erythrocyte inhibitor									
Prep. No.‖									
1	10		4/(26)	6	13	10			
2	11		5	10/(56)		5	78	78	
3	14		1/(75)	2	>200/(34)	4	>915/(90)	>100/(64)	
4	10		3	1	12		>100/(64)		
5	16		1(+)	1	143	143	50/(+)	53	
6	10		1(+)	1	10	26	50/(+)	52	
7¶	8		20	1	52	5	52/(82)	52	
8	16		>78	7	>78	30	>78	>78	
9	11	>68	6	1	68	6	>68	>68	
10**	22	>48	48	.2	>48	>48	>48	>48	
11††	20	>50	20	1	>50	>50	>50/(80)	>50	
12††	17	>55	11	.2	>55	>55	>55/(87)	>55	
Guinea pig erythrocyte inhibitor									
1	17	1	1	1	2	1	17	44	2
2‡‡	8		>51	2/(36)	20/(40)	5/(58)	>51/(76)	>51	
Sheep erythrocyte inhibitor									
LR stroma fraction§§			>252	>252	>252	>252	>252	>252	
Ext. 1	21		>221/(0)	>111	>111	>111	>221/(0)	>211/(0)	
2	8		>570	>570	>189	>189	>189	>189	
3‡‡	7		>52	>52	>52	>52	>52	>52	

* All strains except NWS heated to 56°.

† Expressed as % of total hydrolyzable chromogen in substrate. + = identified by chromatography only; 0 = none detected by thiobarbituric acid method. Avg ratio, mg substrate/μg "viral" N = 0.1 (range 0.02-0.16).

‡ Human erythrocyte preparations 1-9, and guinea pig preparations by Bial orcinol method; human preparations 10-12 by thiobarbituric acid method, maximum chromogen being released after hydrolysis in 0.1N H_2SO_4 at 80° for 30 min.

§ Enzyme assays performed at 26°.

‖ No. 1-9 from blood of single donors by method in ref. 3.

¶ 18% of total hydrolyzable NANA released by NDV.

** Sample kindly supplied by Dr. R. H. Kathan.

†† Prepared from stroma from individual donors by method in ref. 11, with modifications.

‡‡ Water soluble fraction from chloroform-methanol insoluble fraction.

§§ μg nitrogen.

between the A2/Jap and RI/5+ strains on the one hand and the RI/5− strain on the other. It might be noted that these 3 viruses possess the most potent and stable neuraminidase activity of any strains thus far studied.

As shown in Table II, essentially the same pattern of dissociation was observed with respect to the inhibitors obtained from erythrocytes. Although a number of preparations appeared to be somewhat limited in range against the several indicators, all were susceptible to attack by active virus. The "purer" materials (human erythrocyte preparations 10, 11 and 12) appeared to be the most restricted of all, being active primarily against FM1. On the other hand, guinea pig receptor substance had a much broader spectrum of activity. Preparations of receptor substance from both human and guinea pig erythrocytes were susceptible to the neuraminidase of all strains of active virus tested. In contrast, analogous material from sheep erythrocytes, although containing appreciable sialic acid, was totally inactive and furthermore was unaffected by concentrated active virus. The possibility that inhibitory activity may have been destroyed or altered during the extraction procedure is minimized by the fact that the crude sheep stroma fraction (LR) from which the final material was derived was also inactive. Such findings may also account for the relative inagglutinability of sheep cells by most myxoviruses at room temperature, with the notable exception of some strains of Asian influenza virus. These latter resemble the RI/5+ strain in that they do not elute readily from chicken erythrocytes. They do, however, elute slowly but completely from sheep erythrocytes, with resulting destruction of receptors. The apparent insusceptibility to viral neuraminidase of the sheep substrate may appear to be inconsistent with some of the previous observations. One can only presume that the hemagglutinin acts independently of neuraminidase, the latter still being necessary for elution from the intact erythrocyte. A slow rate of elution, as noted with some Asian strains, may reflect either inaccessibility to viral enzyme or differing configuration of the mucoprotein receptor substances at the sheep erythrocyte surface.

Summary. A number of mucoids of varying NANA content were found to differ widely in their capacity to inhibit the hemagglutinin of several strains of myxovirus when assayed under standardized conditions. This pattern of inhibition bore no quantitative relationship to the content of NANA of individual mucoids, each of which was found to be susceptible to the neuraminidase of 2 or more strains of active virus. Receptor substance from human and guinea pig erythrocytes inhibited hemagglutination and was susceptible to viral neuraminidase. Stroma and sialic acid-containing receptor-like substances from sheep erythrocytes were neither capable of inhibiting hemagglutination nor susceptible to viral neuraminidase. The implications of these findings are discussed with respect to the separate identities of myxovirus hemagglutinin and enzyme.

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Polycythemia Secondary to a Pheochromocytoma with Production of an Erythropoiesis Stimulating Factor by the Tumor. (26957)

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Polycythemia has been noted in association with various neoplasms including renal tumors (1,2), cerebellar hemangioblastomas (3,4), uterine fibroids (5) and pheochromocytomas (6). Hematologically these patients have shown erythrocytosis with no associated splenomegaly or increases in the white cell or platelet count. The elevated hemoglobin and hematocrit returned to normal following resection of the tumors. An erythropoiesis stimulating factor (erythropoietin) has been demonstrated in a saline extract of a renal carcinoma (7) and in the fluid obtained from renal cysts (7,8) and cystic cerebellar hemangioblastomas in patients with associated erythemia (4).

In this report we describe a patient with polycythemia associated with pheochromocytoma tumors and demonstrate production of an erythropoiesis stimulating factor by the tumor.

Case summary. D. R., a 10-year-old white boy, was admitted to the Univ. of Maryland Hospital with a complaint of headache. Six months prior to admission the patient developed progressively severe headaches, drenching night sweats, and a 12 lb. weight loss. Two weeks prior to admission the blood pressure was elevated to 170/140 mm Hg.

On admission the patient was markedly plethoric. Blood pressure was 180/110 mm Hg in the upper extremities and 210/160 mm Hg in the lower extremities. A round non-tender walnut-size mass was found in the left flank just under the costal cage. There was no splenomegaly and the remainder of the physical examination was negative.

Laboratory data. The hematocrit was 65%, hemoglobin 21 g %, red cell count 8.3 million/mm^3, leucocytes 8000/mm^3, and platelet count 745,000/mm^3. The bone marrow showed marked erythroid hyperplasia. Total red cell volume was 40 cc/kg, 14 cc/kg above estimated normal value. Arterial oxygen saturation was 93%. Urinary catecholamine excretion determined on 2 occasions gave results of 792 and 1014 units.

At operation 4 tumor masses were removed, 2 from the left adrenal, one from the bifurcation of the aorta and one from the right adrenal. These tumors were microscopically identified as pheochromocytomata.

The patient gradually became normotensive and has been free of symtoms for the year following surgery. The blood data were as follows: hemoglobin 13.8 g %, hematocrit 39%, red cell count 4.65 million/mm^3, and red cell mass was 26 cc/kg. Catecholamine excretion was 4 units.

TABLE I. Erythropoietin Assay.

Material assayed*	Material assayed per inj. per rat	RBC Fe^{59} uptake % at 16-18 hr	Reticulocyte per 1000 RBC
Albumin control (12)	2 ml of 7%	6.0 ± 1.4	2 ± 1
Normal serum (12)	2 ml	6.4 ± 1.4	3 ± 1
Patient's " (12)	2 "	10.3 ± 3.5	—
Saline extract of tumor, non-dialyzable fraction (14)	.4 g of tumor tissue	15.2 ± 4.2	19 ± 5
Saline extract of tumor dialyzable fraction (14)	*Idem*	6.2 ± .9	—
Ether extract of tumor (6)	"	6.8 ± 2.1	—

* No. of rats utilized in assay is shown in parentheses.

Special studies. Methods. The erythropoiesis stimulating activity of the patient's serum and of a saline and lipid extract of a homogenate of the pheochromocytomata tumors was studied by a modification of the fasted rat assay for erythropoietin of Fried *et al.* (9). Normal serum, albumin, adrenaline*, cortisone† adrenocorticotropic hormone‡, testosterone§, and a saline extract of dog adrenal were assayed simultaneously as controls.

Tissues to be assayed were weighed, ground into a fine suspension in a Potter Elvejhem homogenizer and either saline or ether was added. After centrifugation the supernatant fluid was removed and used for assay.

Groups of 6 to 14, 140-160 g, female Sprague Dawley fasted rats were utilized as the recipient animals. The material to be assayed was injected subcutaneously 24 and 48 hours after onset of the fast and 0.5 μc of Fe^{59} was injected intraperitoneally at 80 hours. The rats were bled 16 to 18 hours after administration of the isotope and the hematocrit and incorporation of Fe^{59} into red blood cells determined assuming a blood volume of 5% of the body weight for all recipients.

Results. The results of the assay of erythropoiesis stimulating activity are shown in Tables I and II. On subcutaneous injection of the saline extract of tumor tissue, there was a significant stimulation of erythropoiesis in the fasted rat as shown by an elevated Fe^{59} incorporation into red cells and reticulocytosis compared to animals that received 2 ml of normal plasma or 5% albumin solution. The serum removed from the patient prior to operation also showed some erythropoiesis stimulating activity. The activity appears to be non-dialyzable and not soluble in ether. Groups of 6 rats given adrenocorticotrophic hormone, adrenalin, cortisone, testosterone, and a saline extract of normal dog adrenal had an 18 hour Fe^{59} incorporation comparable to normal serum. This differentiates the known erythropoietically active substances related to the adrenal from the active substances in the pheochromocytoma extract. No significant differences were noted in the mean hematocrit of the various groups studied, excluding the possibility that any of the agents administered were hemolytic.

TABLE II. Erythropoietin Assay.

Material assayed*	Material assayed/inj./rat	RBC Fe^{59} uptake % at 16-18 hr
Saline control (12)	2 ml of .9%	5.0 ± 1.4
Saline extract of normal dog adrenal (6)	.4 g of tissue	4.2 ± .9
Adrenalin (6)	1:1000 (.2 ml)	6.2 ± 1.4
Cortisone (6)	25 mg	5.6 ± 1.0
Testosterone (6)	25 "	5.1 ± 1.4
Adrenocorticotrophic hormone (6)	40 units	4.8 ± 1.2

* No. of rats utilized in assay is shown in parentheses.

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* Parke, Davis & Co.

† Merck Sharp & Dohme Co.

‡ Wilson Laboratories, Inc.

§ Eli Lilly & Co.

Discussion. Polycythemia in childhood is most commonly associated with anoxia due to cardiac or pulmonary disease. Rare cases of congenital familial polycythemia and polycythemia vera have been reported in this age group (10). These causes of polycythemia may be excluded in the present case in view of the normal arterial oxygen saturation and the hematological response to tumor resection. Demonstration of an elevated total red cell volume indicates that this case represents true rather than relative or stress polycythemia where there is a normal total red cell volume but a significantly reduced plasma volume (11). The present studies suggest that the polycythemia seen in association with the pheochromocytoma tumors in this patient is the result of production by the tumor of a substance that stimulates erythropoiesis. The active material appears to be stable on freezing and thawing, not lipid soluble and nondialyzable.

Carnot and Deflandre (12) first suggested that red cell production by the marrow was controlled by a circulating stimulating substance. Recent studies from various laboratories have demonstrated the presence of such a hormone, erythropoietin, in serum and urine of anoxic and anemic animals and man (13). The work of Jacobson *et al.* (14) suggests that the kidney is the major or even sole site of production of erythropoiesis stimulating factors. Gurney (7) and Nixon *et al.* (8) have demonstrated production of erythropoiesis stimulating factors by renal cysts and a renal tumor in patients with associated polycythemia.

Erslev (15) and Mirand *et al.* (16) contend that there are extra-renal sites of erythropoietin production. The present study confirms this view indicating that erythropoiesis stimulating factors can be produced in extra-renal tissue (a pheochromocytoma) at least in pathological states.

Summary. 1. Erythropoiesis stimulating activity has been demonstrated in the saline extract of 4 pheochromocytoma tumors and the serum from a 10-year-old boy with hypertension and polycythemia. 2. The erythropoietically active material was nondialyzable and not lipid soluble.

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Stimulation of Platelet Production by Serum of Platelet-Depleted Rats. (26958)

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Investigations into the effects of platelet depletion on peripheral platelet counts and on megakaryocytes of the bone marrow (1-4) have demonstrated that such thrombopenic measures bring about, after a delay of a day or two, an increased rate of entry of platelets into the peripheral circulation, platelet numbers rising to values more than 1½ times normal levels. Both the delay in response and the changes seen in the megakaryocytic series have indicated that the response is caused by an increased production of platelets rather than by a quick release from a pool. Thus, it appears that a subnormal level of platelets in the circulation triggers an increased production of these blood elements. It has also been reported that normal human serum injected into a splenectomized thrombopenic patient (5), and extracts of normal human serum injected serially into rabbits (6) produced an increase in number of circulating platelets, presumably by stimulating platelet production. An increase in production might result either by the action of a stimulating agent or by removal of an inhibitor. If a stimulating agent were responsible, it should be present in greater-than-normal amounts in the blood of platelet-depleted individuals.

Therefore, we have assayed in untreated and in splenectomized rats serum obtained from platelet-depleted rats, using peripheral platelet counts as the measure of effect. We observed increases in platelet counts to about 150% of control values 5 days after serum injection.

Materials and methods. We used male Sprague-Dawley rats for serum donors and for assay animals; the latter weighed 350 to 450 g. Reduction in number of platelets of serum donors was accomplished in either of 2 ways. One method was to remove by cardiac puncture 40% of the blood volume (taken as 5% of body weight) at about 9 a.m. and another 25% at about 2 p.m. After each withdrawal, a volume of Tyrode's solution equal to half the amount of blood removed was injected intraperitoneally. By this method, as much as 50% of the circulating platelet mass was removed (4). A second method of platelet depletion was to inject rat-platelet antiserum that was made in rabbits. Usually 2.5 ml of a 5% suspension of rat platelets were injected intravenously into rabbits twice a week for 3 weeks, and serum was collected about 10 days after the last injection. In some instances, platelets were mixed with Freund's reagent and injected into footpads and axillary and inguinal regions. Similar titers against rat platelets were obtained by both methods. Serum collected from the platelet-injected rabbits was heated at 56° C for 45 minutes and then absorbed 3 times with an equal volume of homologous rat erythrocytes to remove anti-rat antibodies not specific to rat platelets. Red cells were prepared for this purpose by defibrinating normal rat blood to remove platelets (4). Intraperitoneal injection of 0.25 ml of this antiserum reduced platelet counts in most rats to less than 10% of normal values.

Approximately 24 hours after the initial bleeding or after injection of rat-platelet antiserum, the rats were anesthetized with ether and bled from the abdominal aorta into siliconized syringes. The yield was 3 to 4% of body weight collected in about 2 minutes. This blood was expressed into 15-ml centrifuge tubes, allowed to clot, then centrifuged. The serum was pipetted to

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* Operated by Union Carbide Corp. for U. S. Atomic Energy Commission.

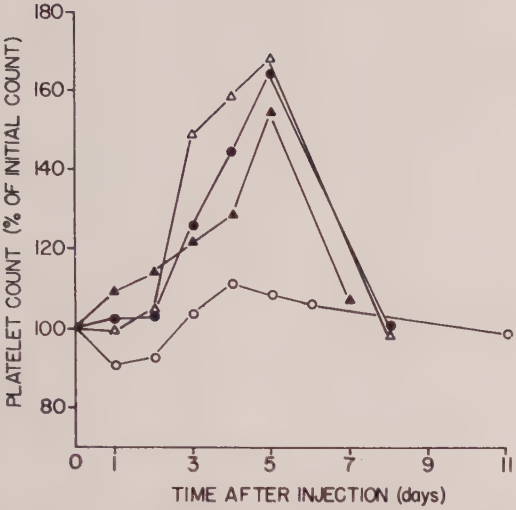

FIG. 1. Peripheral platelet counts of rats inj. with serum of bled donors. Counts were plotted as a percent of the initial count of each rat (Initial Count = 100%). *Active serum*—△, 4 × 12 ml (N = 2); ●, 4 × 6 ml (N = 2); ▲, 4 × 3 ml (N = 4). *Control serum*—○, 4 × 6 ml (N = 5).

other tubes and frozen for storage. Serum recipients were injected twice daily, morning and afternoon, for 2 days. Serum was injected subcutaneously in amounts of 1 to 12 ml per injection, or a total of 4 to 48 ml per recipient.

Peripheral platelet counts were taken before the initial injection and at intervals thereafter. In some experiments counts were taken daily and in others only at 5 days after initial injection. Blood samples, taken in a red cell pipette from a puncture in the lateral vein of the hind limb, were diluted with ammonium oxalate. Counts were made by the direct phase microscope method of Brecher and Cronkite (7). In some experiments, total white blood cell counts were also made before the initial injection of serum and again 5 days later.

Results. Injection of serum from platelet-depleted rats caused increases in peripheral platelet counts as high as 167% of the initial counts (Fig. 1). No appreciable change took place during the first 48 hours after the injections (Table I). The increase was rapid, however, between 48 and 120 hours. The peak count occurred at 5 days, and by the 7th or 8th day platelet numbers had returned to normal levels. Equal amounts of serum from untreated donors did not produce such an effect.

The results (Fig. 1) suggest that the 4 × 6-ml dose (4 injections of 6 ml each) produced a greater response than the 4 × 3-ml dose. Additional groups of rats were injected with these 2 doses of active serum, obtained either from bled donors or from donors injected with rat-platelet antiserum, and the 5-day gains in numbers of platelets were determined (Table II). The data were examined using the covariance analysis method, with gain as the variable and initial platelet count as the covariable; the level of rejection was taken as 5%. Responses to the 2 dose levels were significantly different. However, at each dose level it made no difference whether the active serum was obtained from bled or from antiserum-injected donors.

TABLE I. Early and 5-Day Platelet Response to Serum of Bled Rats (4 × 6 ml).*

Hour	No. of rats	% of initial count	Platelet count range ($\times 10^{-3}/mm^3$)
0	6	100	730-1085
1	6	92	620-1120
8	6	105	745-1240
18	3	102	990-1055
24	3	89	705- 895
36	3	94	870- 970
48	3	97	780- 945
120	6	158	1230-1770

* Platelet counts were taken on all recipients twice before treatment (at –24 and 0 hr) and at 1, 8, and 120 hr after treatment. At other intervals, counts were taken on alternate halves of the total group, as indicated.

TABLE II. Gain in Platelet Count at 5 Days; 2 Doses and 2 Sources of Active Serum.

	Recipients		
Treatment of donors	Dose (ml)	No. of rats	Mean platelet gain ($\times 10^{-3}/mm^3$)
Bleeding	4 × 3	16	348
”	4 × 6	31	473
Antiserum	4 × 3	7	209
”	4 × 6	11	427

TABLE III. Comparison of Platelet Response of 3 Untreated and of 3 Splenectomized Rats to Serum of Bled Rats (4 × 3 ml).*

	Splenectomized			Untreated		
	Platelet count ($\times 10^{-3}$/mm^3)			Platelet count ($\times 10^{-3}$/mm^3)		
Day	Mean	Range	% of initial count	Mean	Range	% of initial count
0	915	735-1050	100	1008	885-1105	100
5	1330	1255-1435	146	1343	1235-1475	133

* Platelet counts were made twice on both days, 0 and 5, and the 2 counts from each rat were averaged. Range includes all counts.

Splenectomized rats were also used as assay animals. The response to active serum may have been somewhat greater in splenectomized than in normal rats (Table III), but the data are not conclusive.

We also found that serum of bled rats that was kept frozen for 50 days or was lyophilized retained its platelet-stimulating activity.

White blood cell counts were not altered 5 days after injection of active serum that produced increases in platelet numbers.

Discussion. The results demonstrate that subcutaneous injection of large amounts of serum from platelet-depleted rats into normal or splenectomized assay animals produced a substantial increase in number of platelets in the recipients' peripheral circulation. The increase seems not to be the result of a release of platelets from a platelet pool, since it did not commence until about 2 days after we began the injections of active serum. Experiments on the effects of bleeding on megakaryocyte and platelet response in rats also indicated the absence of a rapidly mobilizable pool of platelets (3,4).

The increase in number of platelets in circulation to levels higher than normal between the 2nd and 5th days indicates that platelet input, and thus platelet production, was greater than normal during this period (or that survival time of platelets was temporarily lengthened). Thereafter, the rate of platelet production apparently declined since platelet numbers returned to normal levels on the 8th day. Since we have reported that platelet life span in Sprague-Dawley rats is about 4.5 days (8), the rapid drop suggests either that some of the platelets produced between the 2nd and 5th days had a shorter than normal survival time in the circulatory system, or that the rate of platelet production was temporarily reduced below normal between the 5th and 8th days. Cronkite (9) showed that transfusion of large numbers of platelets into rats caused peripheral platelet counts to fall to subnormal levels over a period of several days, presumably by inhibiting production.

The mechanism responsible for increased platelet production is not yet known, but we assume that one or more chemical agents play a mediating part. The increased production that occurs after removal of platelets from the circulation (3, 4) could result from removal of an inhibitor to production, possibly residing in or emanating from platelets (9). Theoretically, such a substance could either directly inhibit megakaryocytic cells from producing platelets or could inhibit elaboration of an agent that is stimulatory to megakaryocytic cells. The platelet increase seen after injection of active serum seems more readily attributable to the presence of a stimulatory agent in the donor serum. Thus there may be a balance in normal individuals between an inhibiting agent that derives from platelets and a stimulatory agent, the source of which is not presently known. This type of humoral balance occurs commonly, as, for example, between thyroid hormone and thyroid-stimulating hormone.

An alternative mechanism can be imagined, namely that a diminution in number of platelets, rather than in concentration of an inhibitor deriving from platelets, brings about some functional imbalance that in turn is responsible for initiating elaboration of a stimulatory agent.

We believe that serum of platelet-depleted rats contains an agent that stimulates production of blood platelets. This agent might act on the megakaryocyte compartment of the bone marrow and spleen either by increasing the rate of maturation of megakaryocytes, or by increasing the rate of cell division among the earlier cells in the megakaryocytic series, or both. Examination of the effects of platelet depletion by bleeding on the total and differential counts of cells of the megakaryocytic series suggests that both processes may play a part (4).

The lack of response to similar quantities of serum from untreated donors shows that the observed platelet response is not due simply to injection of relatively large amounts of serum.

It is interesting that an increase to a level of about 150% of normal platelet numbers usually constituted the maximum response to injection of active serum. Perhaps the marrow is not capable of producing any greater short-term response, or an opposite regulatory action (platelet-derived inhibitor?) may come into play by the time these platelet levels have been reached.

Spector has been engaged in similar experiments with rabbits. The results (10), presented concurrently with ours on rats (11), demonstrated that serum from donor rabbits that had been depleted of platelets by specific antiserum or by treatment with Myleran produced an increase in platelets when injected intravenously into untreated or splenectomized rabbits.

Summary. Four subcutaneous injections, given during a 2-day period, of serum from platelet-depleted rats into untreated or splenectomized rats caused an increase in the number of circulating platelets to as much as 167% of the initial count. The rise in peripheral platelet count began 2 days after the first injection, reached a peak at 5 days, then declined to control values at 8 days. Equal amounts of serum from untreated donors did not produce this response. The results indicate that the active serum contains an agent (thrombopoietin) that stimulates production of blood platelets.

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Effects of Salicylate on Prophylactic Counter-Irritation.* (26959)

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A pre-existing inflammation produced by mechanical (1) or chemical (2) irritation has been shown to inhibit partially the response elicited by a subsequent irritation. It has been found in rats that when $AgNO_3$ is used as the chemical irritant, the maximum inhibition of inflammation, as evaluated by the edematous response, occurs when the initial irritation precedes the second irritation by 12-15 hours (3). Because of the extensive use of salicylates in treatment of inflammation, this study was undertaken to evaluate their effects on this prophylactic counter-irritation mechanism.

Materials and methods. The test sites used in this study were the ankles of male albino rats (300-450 g). Chemical irritation was accomplished by injection of 0.10 ml of 1% silver nitrate into the joint under light ether anesthesia (4). Salicylate administration was accomplished by introducing 50 mg of sodium salicylate in 2 ml of water via stomach tube. The edematous response was evaluated by measuring to the nearest millimeter the circumference of the leg immediately superior to the ankle joint. An analysis of variance has shown that there is no significant difference between investigators or repeat measurements in this laboratory. For comparative purposes and ease in presentation, the percent increments in circumference were calculated.

During this study 2 series of experiments were performed. Since statistical analysis ("t" ratios) showed no significant differences between the 2 series, they have been combined for economy of presentation. The animals were divided into the following treatment groups:

A—only the test ankle was irritated (control group);
B—sodium salicylate was administered 1 hour prior to irritation of the test ankle;
C—the opposite ankle was irritated 15 hours prior to irritation of the test ankle;
D—sodium salicylate was administered 16 hours prior, and the opposite ankle was irritated 15 hours prior to irritation of the test ankle; and
E—the opposite ankle was irritated 15 hours prior, and sodium salicylate was administered 1 hour prior to irritation of the test ankle.

Results. In Table I are presented the mean percent increases in circumference of the test ankles following irritation with $AgNO_3$. For the control group (A) the peak response (ca. 55%) was reached within 12 hours and was maintained through the second day; thereafter the swelling subsided and the ankle regained its initial size in 2 weeks. Administration of sodium salicylate one hour prior to irritation of the test ankle resulted in a marked inhibition of edema. The amount of swelling for this treatment (B) was significantly less ($P < .01$) than that occurring in the control group at the 12, 24, and 48 hour determinations, which as noted above correspond to the period of maximum swelling.

In the prior-irritation group (treatment C), in which the opposite ankle was irritated 15 hours previous to injection of the test ankle, the ankle swelling was much less than in the control group. The differences were significant at the 6, 12, 24, 48, and 120-hour determinations. Also, this prior-irritation group exhibited less mean swelling than the salicylate-treated group, but the

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TABLE I. Effects of Prior Treatment with Sodium Salicylate and/or Prior Irritation on Percent Increments of Ankle Edema.

Treatments	n	Hours after irritation of test ankle with $AgNO_3$							
		6	12	24	48	72	120	168	336
A. None	10	44 ± 5*	55 ± 5	55 ± 4	56 ± 7	48 ±10	42 ± 9	27 ± 9	2 ± 2
B. Na sal. (–1 hr)	10	37 ± 7	44 ± 6	45 ± 8	44 ± 8	40 ±15	34 ±15	22 ±13	3 ± 4
C. Prior irrit. (–15 hr)	21	26 ± 9	37 ±10	42 ± 8	40 ± 8	38 ± 9	26 ±11	16 ±10	1 ± 2
D. Na sal. (–16 hr); prior irrit. (–15 hr)	21	38 ±11	50 ± 8	56 ±10	53 ±11	46 ±10	37 ±15	25 ±14	4 ± 4
E. Prior irrit. (–15 hr; Na sal. (–1 hr)	21	28 ±11	38 ±11	41 ± 8	40 ±11	34 ±19	31 ±15	24 ±14	2 ± 5

Treatment comparisons showing significant differences ($P < .01$)†

A *vs* B	12, 24, 48 hr	B *vs* C	6 hr	C *vs* D	6, 12, 24, 48, 72, 336 hr
A *vs* C	6, 12, 24, 48, 72, 120, 168 hr	B *vs* D	24 hr	C *vs* E	—
A *vs* D	—	B *vs* E	—	D *vs* E	6, 12, 24, 48 hr
A *vs* E	6, 12, 24, 48 hr				

* Mean ± stand. dev.

† "t" ratio.

difference was significant at the 6 hour reading only. When salicylate was administered prior to irritation of the opposite ankle (treatment D), thereby inhibiting the "inflammatory" response of the first ankle, the test ankles swelled nearly as much as those of the controls and differed as much from group C as did the controls. In those rats receiving salicylate one hour prior to irritation of the test ankle (treatment E), the opposite ankle having been irriated 15 hours previously, the amount of edema was less than that of the controls; the difference was significant at 6, 12, 24 and 48 hours. The amount of swelling, however, was not significantly less than that obtained with salicylate (treatment B) or prior-irritation (treatment C) alone.

Discussion. As we reported previously, the maximum inhibition of edema in the test ankle occurs when the prophylactic counter-irritation (PCI) precedes by 12 to 15 hours the irritation of the test ankle. In this study, PCI inhibited the edematous response of the test ankle to a slightly greater extent than did sodium salicylate. This, however, is not a significant finding since we have noted greater inhibition of swelling with higher doses of sodium salicylate than were used here. This substantiates the reports of others (4,5). On the other hand, a greater inhibition of the swelling might occur if the degree of inflammation of the first ankle was increased by injecting a larger quantity of irritant. The dose of 0.10 ml of 1% $AgNO_3$ was chosen because with larger quantities sloughing and visible necrosis often resulted.

Administration of salicylate shortly before irritation of the test ankle did not augment the inhibition produced by PCI. One explanation for the lack of summation is the possible interference of one mechanism with the other. An alternative explanation is that both PCI and salicylate utilize essentially the same terminal mechanisms with the result that under the conditions of this experiment either treatment alone leads to near exhaustion of the anti-edema mechanisms. Laden et al (1) have shown that the "protection" afforded by PCI is also present in adrenalectomized and hypophysectomized animals. Further, Kelemen (5), subscribing to the permissive theory for corticoid activity, reports that although corticoids are necessary for the anti-edema action of salicylates, the latter do not operate through the hypophyseal-cortical axis. These observations, therefore, rule out the hypophyseal-cortical axis as a common mechanism.

Of even more interest is the effect seen when salicylate was given just prior to PCI. Here the inhibition of the edematous response usually produced by PCI was markedly reduced, if not lost all together. The interference by salicylate upon the PCI mechanism, as mentioned above, is one possibility for explaining this effect. An alternate possibility of this salicylate effect is that by reducing the inflammatory response to the first irritation the stimulus to the counter-irritation mechanism is sufficiently reduced as not to alter the second inflammation under the conditions of this study.

The existing evidence suggests that PCI leads to production of a generalized hyporeactivity to irritation. Local hyporeactivity at the site of irritation has been established (6,7,8,9). By altering or blocking the PCI mechanism, salicylates may allow a more rapid return to the initial reactivity. However, until the nature of this "protection" against inflammation afforded by PCI is established, it is difficult to evaluate the salicylate effect in this study.

Summary. The effects of sodium salicylate on prophylactic counter-irritation, accomplished by irritating the opposite ankle 15 hours prior to the test ankle, were studied in rats. It was found that: (1) The capacities to inhibit edema by prophylactic counter-irritation and sodium salicylate were similar; (2) Sodium salicylate administered 1 hour prior to irritation of the test ankle did not increase the degree of inhibition produced by prophylactic counter-irritation; and (3) Sodium salicylate given 1 hour prior to the prophylactic counter-irritation caused the latter's anti-edema effect to be lost.

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Species-Related Antigens of Mammalian Cell Strains as Determined by Immunofluorescence.*† (26960)

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In the development of characterizing procedures for cultured cell strains, identification of their species of origin is of primary importance, especially since it is commonly known that some cell strains have become contaminated with others of diverse parentage. This problem has been approached in several ways, including chromosomal analyses(1), virus susceptibility(2), and immunologic identification(3-4). In an effort to identify cell strains in a manner analogous to the systematic serology long employed in

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† Presented in part at April 1961 meeting of Fed. Am. Soc. Exp. Biol.

TABLE I. Cell Strains Used for Production of Labeled Antisera.

Cell strain	*Species and tissue of origin*	*Characterizing properties*
Detroit 6*	Human, sternal marrow	Epithelial-like, heteroploid, virus susceptibility
HeLa 229†	Human, CA of cervix	idem
Detroit 504*	Human, teratoma of testes	Fibroblast-like, diploid, virus susceptibility
LLC-MK$_2$‡,**	Monkey, kidney	"Epithelial-like," virus susceptibility
S-180§	Mouse, sarcoma 180	"Fibroblast-like," tumorigenic
16F-FAF 28‖	Chinese hamster, peritoneal elements	"Fibroblast-like," karyology

* Cell strains originated in these laboratories.
† Provided by Dr. J. T. Syverton, Univ. of Minnesota, Minneapolis.
‡ " " Dr. R. N. Hull, Eli Lilly Research Laboratories, Indianapolis, Ind.
§ " " Dr. G. E. Foley, Children's Cancer Research Foundation, Boston, Mass.
‖ " " Dr. G. Yerganian, " " " " " "
** Previously LLC-MK$_2$ (NCTC 3196, Evans), cultivated herein in serum medium.

microbiology, this report describes the development of an immunofluorescence method for rapid and specific identification of cultured mammalian cells.

Materials and methods. Cell strains. A variety of mammalian cell strains originally derived from several different animal and tissue sources(5-7) were selected for preparation of antisera. These strains, representing 4 animal species are listed in Table I with information pertinent to their sources and characteristics. These and several additional types of cell cultures were used for the fluorescent antibody tests, namely, Detroit-508, a fibroblast-like strain derived from human kidney; CCRF 228 and 232‡(8), strains derived from mouse lungs (the former from a C57 BL/6 and the latter from a DBA/1 mouse); and primary cultures of Rhesus monkey kidney cells.

Preparation and assay of antisera. The cell strains listed in Table I were used to immunize guinea pigs according to the procedure of Brand and Syverton(3). It was felt that antisera prepared in this manner could be used to verify species origin of cells by their ability specifically to agglutinate erythrocytes of the homologous species(3). It was presumed that such hemagglutinating antibodies could be used as a guide for the efficiency of subsequent conjugating procedures. The various cell strains were cultured as monolayers in 6-oz. prescription bottles, employing Eagle's minimal essential medium(9) and supplemented with 10% human serum for human-derived strains and 5% calf serum for the others. Twenty-four hours prior to harvesting, all monolayers grown in medium with human serum were washed, then re-fed with medium containing 5% calf serum.

To harvest cells, all monolayers were rinsed once with serum-free medium, the cells removed from the glass by scraping, and the dispersed cells washed in 2 additional changes of serum-free medium. Total cell yields from 6 replicate cultures, amounting to 6 to 8 × 10^7 cells, were suspended in 6.5 ml of serum-free medium. Of this, 3.5 ml was emulsified with equal parts of Freund's complete adjuvant. Each of 6 guinea pigs were injected subcutaneously at 4 sites on the abdomen, using 0.25 ml of the emulsion at each site. Simultaneously, each animal received 0.5 ml of cell suspension (without adjuvant) intraperitoneally. The combination of injections was equivalent to approximately 1 × 10^7 cells per animal. Animals were bled by cardiac puncture before, and 4 weeks after injections. In some instances, attempts were made to enhance the antibody titers (specific hemagglutinins) by weekly intramuscular injections of cell suspensions but no appreciable differences could be observed either in titers or specificity of the antisera. The sera (inactivated at 56°C. for 30 min-

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‡ Provided by Dr. G. E. Foley, Children's Cancer Research Foundation, Boston, Mass.

TABLE II. Hemagglutination Characteristics of Various Cell Antisera.

	Hemagglutinin titer * Whole serum						Hemagglutinin titer *† after conjugation with Fluorescein Isothiocyanate					
Species Erythrocytes	Detroit -6	HeLa	Detroit -504	LLC-MK$_2$	Mouse S-180	Hamster	Detroit -6	HeLa	Detroit -504	LLC-MK$_2$	Mouse S-180	Hamster
Human	512	256	1024	64	64	32	32	32	16	0	4	0
Monkey (Rhesus)	32	128	32	512	8	0	0	8	0	16	0	0
Mouse	0	0	4	4	16	32	0	0	0	0	0	0
Hamster ‡ (Chinese)						64						0
Rabbit	4	0	0	0	0	4	0	0	0	0	0	0
Guinea Pig	0	0	0	0	0	0	0	0	0	0	0	0
Sheep	0	0	0	0	0	0	0	0	0	0	0	0

* Reciprocal of dilution.

† Not corrected for dilution effects.

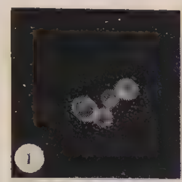
‡ Hamster RBC spontaneously agglutinated with most guinea pig sera.

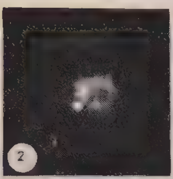
utes) from animals inoculated with a given cell strain were assayed, both individually and in pools, by determination of hemagglutinins for erythrocytes corresponding to the species of origin of the cell strains, according to the procedure of Brand and Syverton(3). The hemagglutinin titers of the various antiserum pools are shown in Table II. The species specificity of the antisera to the cell strains of human origin (Detroit-6, HeLa, Detroit-504), and the cell strain of monkey origin (LLC-MK$_2$), as well as the common antigenic component between the 2 species (3) are evident. On the other hand, antisera to the mouse and Chinese hamster strains yielded inconclusive results with regard to hemagglutinins.

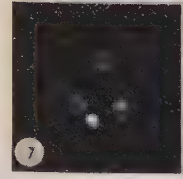
Preparation of fluorescent antibodies. The antisera to be conjugated were fractionated either with half saturated ammonium sulfate or 26% sodium sulfate and dialyzed by standard procedures. Globulins thus obtained were conjugated with fluorescein isothiocyanate (purified, obtained from Baltimore Biological Laboratories), by adding the powdered dye to the globulin solutions at pH 9 with stirring in an ice bath and followed by dialysis against phosphate buffer, pH 7.2(10). The conjugates were stored frozen until used.

Hemagglutinin titers of the conjugates are illustrated in Table II. The conjugation procedure resulted in a general loss in hemagglutinin titer, but the specificities of the conjugates as compared to the whole sera appeared to be retained. In addition, whenever, the serum hemagglutinin titers were low, the corresponding conjugates exhibited little or no hemagglutinin.

Staining procedure. The cells of monolayer cultures were dispersed with 0.25% trypsin solution and the resulting cell suspensions were washed 3 times with buffered saline, pH 7. An alternative procedure involved preservation of cell suspensions(11) by slow freezing in glycerol-serum media, storage at —190°C., rapid thawing, and 4 subsequent washings. The latter method proved to be valuable by eliminating the necessity for preparation of large numbers of cultures of various strains and their possible cross-contamination just prior to staining, while yielding identical results. Each cell suspension was brought to approximately 2% concentration by volume in buffered saline, pH 7.4. One-tenth ml of cell suspension was mixed with 0.1 ml of labeled antibody in the wells of non-wettable plastic slide plates which were then placed in a humidity chamber on a rotary shaker and agitated for 30 minutes at room temperature. Stained suspensions were then transferred to small tubes, and washed with 3 changes of buffered saline. Following the last wash, the supernate was decanted, and the cells resuspended in the fluid that remained. One drop of this suspension was placed on a slide (not exceeding 1 mm in thickness), covered with a #1 coverslip, gently pressed with blotting paper, and sealed with clear finger-nail lacquer. Such preparations were examined within 2 hours by fluorescence microscopy,







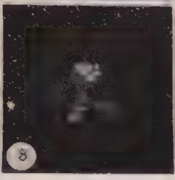


FIG. 1. Detroit-6 cells stained with anti Detroit-6 conjugate.

FIG. 2. LLC-MK_2 cells stained with anti Detroit-6 conjugate.

FIG. 3. Chinese hamster cells stained with anti Detroit-6 conjugate.

FIG. 4. HeLa cells stained with anti HeLa conjugate.

FIG. 5. Detroit-504 cells stained with anti HeLa conjugate.

FIG. 6. Detroit-6 cells stained with anti HeLa conjugate.

FIG. 7. S-180 cells stained with anti S-180 conjugate.

FIG. 8. LLC-MK_2 cells stained with anti S-180 conjugate.

FIG. 9. Chinese hamster cells stained with anti Chinese hamster conjugate. (All photomicrographs are at 300 × total magnification)

using a BG-12 pass filter and an OG-1 barrier filter. This staining procedure was suggested by the work of Cohen, *et al.*(13) who demonstrated blood group antigens by peripheral staining of erythrocytes with fluorescein-labeled antibodies.

Observations and results. The interpretations of the staining reactions were based entirely on presence or absence of *peripheral* fluorescence of the cells. Fig. 1 illustrates a group of cells stained with homologous labeled antibody, and provides an example of what will be referred to as "specific" or "positive" staining. Additional examples of specific staining of the various cell strains are shown in Fig. 4-7 and 9. The intensities of the positive reactions varied to some extent from conjugate to conjugate. Brilliant staining was encountered when labeled Detroit-6, HeLa, or Detroit-504 antibodies were applied to Detroit-6 and HeLa cells (Figs. 1, 4, 6). However, each of these labeled antibody preparations produced a thin but well-defined periphery of fluorescence with Detroit-504 cells (Fig. 5), although the latter was difficult to reproduce photographically. At the other extreme, 2 of the conjugates, namely anti S-180 and anti Chinese hamster, produced weaker but nevertheless specific peripheral staining with their homologous cell preparations (Fig. 7, 9). The conjugates also exhibited some variation with regard to the number of cells that appeared unstained in otherwise positive homologous antibody-cell systems; an estimated 1-5% of the cells did not exhibit peripheral fluorescence in a given preparation. The incidence of the latter appeared to be inversely related to intensity of the staining. Care was taken to distinguish true peripheral staining of individual cells from that which occurred when conjugate was trapped between cells in instances where large clusters or clumps were present.

Negative reactions were defined as those in which no peripheral fluorescence was evident (Fig. 2, 3, 8). In these instances the cells exhibited mottled or solid fluorescent areas that could be distinguished from positive reactions.

Cross-reactions between antibody preparations for cells of human and monkey origin observed in the hemagglutination reactions (Table II) were also evident by staining with fluorescent antibodies. It was important therefore, to attempt selective absorption of the cross-reacting antibodies involved. Absorption with cultured cells was not feasible at the time because of the unavailability of the quantities of cells needed. However, since erythrocytes had been shown to be effective in removing cross-reacting hemagglutinins between these two species(3), it was felt that erythrocytes might also be effective for absorption of those conjugates which exhibited hemagglutinin activity. Thus, the conjugates made against the Detroit-6 and LLC-MK_2 strains were absorbed § with Rhesus monkey or human O negative red cells. The results

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§ The conjugates were absorbed twice with one volume of packed red cells to 2 volumes of conjugate for 30 minutes in the cold.

TABLE III. Effect of Absorption on Cross-Staining Between Fluorescent Antibodies and Cells of Human and Monkey Origin.

	Staining titer * of labeled globulins					
	Unabsorbed		Absorbed			
			with human RBC		with monkey RBC	
Cell strain	Detroit-6	LLC-MK$_2$	Detroit-6	LLC-MK$_2$	Detroit-6	LLC-MK$_2$
Detroit-6 (Human)	8	2	2	0	2	0
LLC-MK$_2$ (Monkey)	4	4	0	2	0	2
Sarcoma 180 (Mouse)	0	0				
CCRF 228 "	0					
CCRF 232 "	0					
Chinese Hamster	0	0				

* Reciprocal of dilution.

are shown in Table III. Although these absorptions eliminated the cross-staining between the fluorescent antibodies of the 2 species, paradoxically, absorption with either species of red cells rendered the staining reactions specific. The explanation for the latter phenomenon is not clear.

In Table IV are shown the staining characteristics of the various absorbed conjugates for cells of homologous and heterologous species origin. The labeled antibodies prepared against the Detroit-6, HeLa, or Detroit-504 strains reacted specifically only with cells of human origin. Similarly, the labeled antibody against the monkey kidney strain was positive not only for that strain, but for primary cultures of monkey kidney cells as well. The S-180 labeled antibodies produced recognizable peripheral staining of S-180 cells, but when tested against the 2 mouse strains of normal tissue origin, the results were somewhat evanescent, possibly because this antibody preparation was weak (Fig. 7). The fluorescent antibodies prepared against the Chinese hamster strain were also specific only for those cells. With the exception of Detroit-508, each combination was repeated at least several times, and where sufficient labeled antibody was available (Detroit-6, HeLa, LLC-MK$_2$), these were used as additional controls with homologous and heterologous cells with each combination tested. There were no instances where discrepancies occurred with the combinations shown in Table IV.

Discussion. Current problems in cell research demonstrate the need for methods that will distinguish cell strains with respect to their species-related antigens. For the purposes of a cell culture collection, it was necessary to develop a practical method for rapid and direct identification of cell strains to be characterized. Of the various immunological studies that have been reported, the work of Brand and Syverton, involving indirect hemagglutination(3), and that of Coombs et al., involving the mixed agglutination technique(4), have offered the most promising approach to this problem. Both of these methods involve detection of species antigens

TABLE IV. Staining Characteristics of Various Fluorescein-Labeled Antisera.

	Staining reactions with labeled antibodies					
Cells	Detroit -6	HeLa	Detroit -504	LLC-MK$_2$ (Monkey)	S-180 (Mouse)	Chinese Hamster
Detroit-6 (Human)	+	+	+	0	0	0
HeLa 229 "	+	+	+	0	0	0
Detroit-504 "	+	+	+	0	0	0
Detroit-508 "	+	+	+	0	0	0
LLC-MK$_2$ (Monkey)	0	0	0	+	0	0
Primary kidney "	0	0	0	+	0	0
S-180 (Mouse)	0	0	0	0	+	0
CCRF 228 "	0	0	0	0	±	0
" 232 "	0	0	0	0	±	0
Chinese hamster	0	0	0	0	0	+

common to both red cells and cultured tissue cells. The experiments described herein suggest that fluorescein-labeled antibodies may provide a means for directly identifying species-related antigens on cell surfaces.

To test the feasibility of the procedure, antibodies were prepared against cell strains whose origins were reasonably certain on the basis of one or more identifiable "marker" characteristics, *i.e.*, morphology, karyology, tumorigenicity, or virus susceptibility spectrum. Moreover, the origins of several of the strains were verified by the hemagglutination method, and all were verified by the results of the fluorescent antibody tests. Nevertheless for preparation of reference antisera, the use of primary cultures might be preferable, principally to obviate questions of source or antigenic changes. The latter is currently under investigation. Although the immunization procedures employed herein involved the use of guinea pigs, the latter were not wholly satisfactory, primarily because of the weak antibody responses to cells of mouse or hamster origin, as measured both by hemagglutinins and by fluorescent antibodies.

The data suggest that hemagglutination and fluorescence may be detecting different groups of antibodies, since specific fluorescence was obtained in the absence of hemagglutination. This made it difficult to assess the efficiency of the conjugation procedures, although it is quite clear that 26% sodium sulfate was unsatisfactory for serum fractionation since marked loss of titer occurred due to dilution effects.

It is likely the fluorescent antibodies in the stained preparations were reacting with antigens at the cell surface, analogous to the agglutination reactions studied by Kite and Merchant(12), and the mixed agglutination reaction of Coombs et al.(4). It should be emphasized that the staining reactions took place on surfaces of live cells in suspension. The use of dried or fixed smears or monolayers was entirely unsatisfactory because dead or non-viable cells stained non-specifically. The cells in the preparations were spherical and it was presumed that the exposed antigenic sites were concentrated over a minimal surface area of the contracted cell in contrast to cells attached to glass where the cell surfaces would be spread out over a much greater area.

Summary. A procedure has been described whereby species-associated antigens were directly identified in cultured mammalian cells by fluorescent antibody techniques. Guinea pig anti-cellular globulin fractions were conjugated with fluorescein isothiocyanate. Dispersed cells were prepared from monolayer cultures or frozen cell suspensions and the resulting wet cell suspensions were treated with the labeled antibodies. Staining specificity was recognized by presence or absence of peripheral fluorescence of the treated cells. Four cell strains derived from human tissues, 3 from mouse, and one from Chinese hamster, reacted specifically with homologous but not with heterologous labeled antibodies. Antibodies to monkey- and human-derived strains exhibited reciprocal cross-reactions that were eliminated by absorption procedures.

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Prolactin Production by Rat Pituitary *in vitro*.*‡ (26961)

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A number of investigators have attempted to cultivate anterior pituitary (AP) *in vitro* and assay for hormone production(1-4). While hormone production by serial propagation of this tissue has met with limited success(5), organ explants of pituitary tissue have produced detectable quantities of ACTH(6) and TSH(7). Prolactin should be an ideal hormone for *in vitro* production by AP tissue, since there are definite indications that removal of CNS influences results in increased prolactin secretion. Thus, (a) transplantation of the AP to the kidney capsule of rats elicits pseudopregnancy and mammary growth(8-10), and (b) induction of a specific hypothalamic lesion in estrogen-primed rabbits promotes lactation(11). The present *in vitro* study demonstrates that AP tissue from rats releases many times more prolactin into culture medium than is normally present in fresh AP, and that pituitary explants from immature, mature and lactating rats show significant differences in prolactin production.

Methods. Female rats of the Carworth CFN and Sprague-Dawley strains provided pituitaries for these experiments. The former consisted of virgin females 3-4 months old, and primiparous lactators 15-17 days postpartum; the latter included 21-30 day old immature females, non-pregnant multiparous females, and primiparous lactators 3-4 days postpartum.

Both the Chen(12) and a modification of the Trowell 1959(13) organ culture method of Fell and Robison(14) were employed. After decapitation, the AP was removed aseptically from each rat and placed on moistened filter paper in a Petri dish. The AP was cut into 6 pieces, each about 2 mm in diameter, placed on rafts of cellulose-acetate or stainless steel mesh, and floated on a nutrient medium in a watch glass inside of a Petri dish. Explants from 2 or 3 AP's were incubated in each dish. Pituitary explants from Carworth rats were cultured on "199" medium containing 2 units Zn-free insulin §, 50 IU penicillin G potassium and .1 mg streptomycin sulfate per ml. Pituitary explants from Sprague-Dawley rats were cultured on Trowell T-8 medium according to the procedure outlined by Merchant et al.(15) Cultures were incubated at 35 ± 1°C in air atmosphere or gassed continuously with 95% O_2-5% CO_2.

Medium cultured in air atmosphere was replaced daily with 1 cc fresh medium, and medium gassed with O_2-CO_2 was replaced every 3 days with 3 cc fresh medium. The collected medium was preserved in a freezer, with the exception of medium from the 1st day of culture from groups 1-4 in experiment 1 (Table 1), which was discarded to remove possible contamination from cellular debris. The medium from each group was lyophilized, dissolved in distilled water, and assayed for prolactin in mature White Carneau pigeons by the intradermal method of Lyons(16) as modified by Reece and Turner(17). Approximately 8 Reece-Turner units are equal to 1 IU prolactin by our method of assay. The 6-day medium from each group in Experiment 1 was combined for assay; in Exp. 2 each 3-day medium was assayed separately and the results were combined for the 9-day period. Some pituitary explants were also assayed at the end of 6 days culture. For controls, the same volumes of incubated "199" medium without pituitary explants were assayed for prolactin activity in 3 separate trials in 13 pigeons, and insulin alone was assayed in 3 birds. At the end of each culture period, most pituitary explants were fixed in Bouin's fluid, sectioned at 6μ, and stained with eosin and hematoxylin. Data were analyzed by the

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** Pre-doctoral fellow, NIH.

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TABLE I. Prolactin in "199" Medium from Cultures of Anterior Pituitaries from CFN Rats.

Group	No. of pits	Type of rat	Days of culture	Atmosphere	No. of Birds per assay	Prol. Units per pit/day
1	6	Mature ♀	2-7	Air	3	.1
2	4	Lactators	2-7	"	3	.4*
3	6	Mature ♀	2-7	"	5	.2
4	3	Lactators	2-7	"	5	.4*
4	3	"	8-14	"	5	.2
5	3	Mature ♀	1-6	O_2-CO_2	5	.9†
6	3	"	1-6	"	5	1.0†
7	3	"	1-6	"	5	1.1†

* Groups 2 + 4 *vs* 1 + 3 : $P<.05$ † Groups 5,6,7 *vs* 1 + 3 : $P<.001$

Kruskal-Wallis sum of ranks test(18).

Results. Neither the 3 control media nor insulin showed prolactin activity. By contrast, all media incubated with pituitary explants contained substantial amounts of prolactin. Pituitary explants from lactators (Table 1, Groups 2 and 4) released 2-4 times as much prolactin per day into the medium as explants from mature females (Groups 1 and 3). When explants from Group 4 were cultured for an additional 7 days (days 8-14), about half as much prolactin was produced as in the previous 6 days. Pituitary cultures gassed continuously with 95% O_2 - 5% CO_2 (Groups 5-7) released 5-10 times more prolactin into the medium each day than cultures exposed to air atmosphere (Groups 1 and 3). These differences are highly significant. The limited number of assays of prolactin content in pituitary explants at the end of a 6-day culture showed much less prolactin than fresh AP tissue at the beginning of culture.

In the 2nd experiment (Table 2), about half as much prolactin was released into the medium each day by AP explants from immature rats (Groups 1 and 4) as by explants from mature rats (Groups 2 and 5). This difference was significant at the 2% level. Pituitary explants from lactating rats (Groups 3 and 6) released about twice as much prolactin into medium as explants from mature females (Groups 2 and 5). This difference was significant at the .1% level. Histological examination of AP explants after 6-7 days of culture in Exp. 1, revealed that tissue exposed to air atmosphere was necrotic with only a thin outer rim of living cells (Fig. 1), while explants gassed with 95% O_2 - 5% CO_2 consisted predominantly of viable cells (Fig. 2). The latter compared favorably in appearance with fresh rat AP tissue (Fig. 3).

Discussion. These results show that AP explants from rats can synthesize and release substantial amounts of prolactin into chemically defined media when cultured *in vitro*. Pituitary prolactin content in a mature female Carworth rat shows a range of .3 - .5 RT units when assayed in mature White Carneau pigeons(19). By contrast, gassed explants in Exp. 1 released 2-3 times more prolactin per AP (.9 - 1.0 RT units) into culture medium each day. In 6 days, each pituitary released

TABLE 2. Prolactin in Trowell Medium from Cultures of Anterior Pituitaries from Sprague-Dawley Rats. (95% O_2-5% CO_2 Atmosphere)

Group	No. of pits	Type of rats	Days of culture	No. of birds per assay	Prol. units per pit/day
1	4	Immature ♀	1-9	9	.1
2	4	Mature ♀	"	"	.2*
3	4	Lactators	"	"	.4†
4	3	Immature ♀	"	"	.1
5	3	Mature ♀	"	"	.2*
6	3	Lactators	"	"	.4†

* Groups 2 + 5 *vs* 1 + 4 : $P<.02$ † Groups 3 + 6 *vs* 2 + 5 : $P<.001$

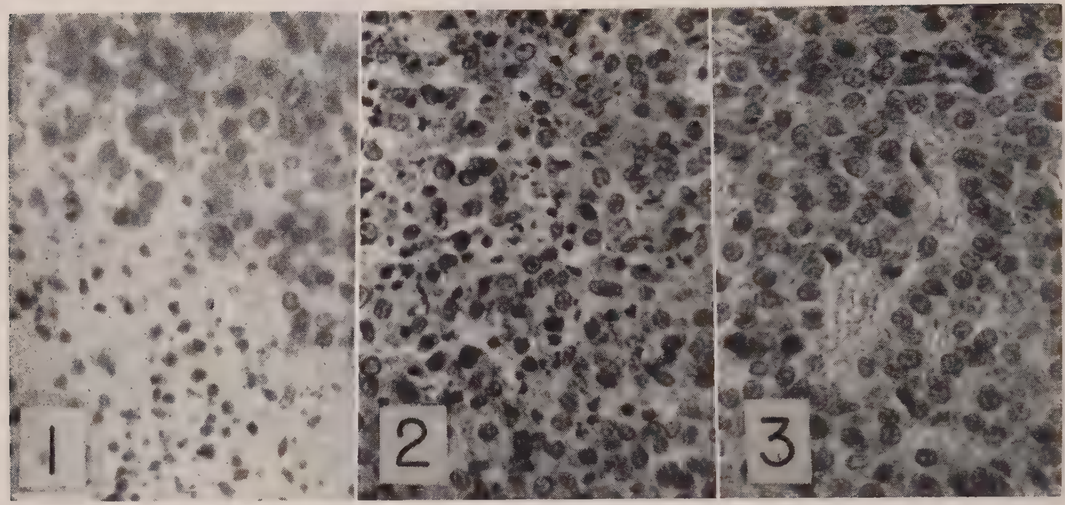

FIG. 1-3. Histological sections from rat anterior pituitary (AP): (1) Explant cultured for 7 days in air atmosphere. Note outer rim of living cells (2) Explant cultured for 6 days in 95% O_2–5% CO_2. Most cells are viable (3) Section from fresh, non-cultured AP. × 440.

about 6 RT units (.8 IU), or 12-18 times as much prolactin as was originally present in the AP. Since some destruction of pituitary cells must have occurred during initial handling and cutting of pituitary explants, potential production of prolactin was probably even greater than noted in these experiments. These data clearly denote active synthesis and release of prolactin by AP tissue, and not mere "leaching" of residual hormone.

Smaller quantities of prolactin were recovered from Trowell as compared to "199" medium when each was gassed with 95% O_2 - 5% CO_2. This is believed to be due to employment of chloramphenicol in Trowell medium which may have interfered with hormone synthesis. The greater viability of gassed as compared to non-gassed AP tissue at the end of 6 days culture was clearly reflected in greater prolactin production. It is of interest however, that we have successfully cultured rat AP tissue for 21 days in air atmosphere with continuous production of prolactin, despite the small area of viable tissue remaining at the end of culture (Nicoll and Meites, unpublished). Although only preliminary assays have been made of AP explants at the end of culture, their relatively low prolactin content suggests very rapid synthesis and release of hormone. Differential staining of AP tissue cultured on Trowell medium for 7 days by one of us (Kahn) indicates that only few atrophic basophils are present and that acidophils predominate. The latter cells are usually associated with prolactin secretion(20).

The present *in vitro* findings corroborate the conclusion that differences in pituitary prolactin content previously reported in immature, mature and postpartum rats(17,21), represent actual differences in secretion rate. Estrogen is believed to be largely responsible for greater prolactin secretion by pituitaries of mature as compared to immature rats, and for the rise in prolactin production at about the time of parturition(21). Administration of estrogen increases pituitary prolactin content *in vivo*(17,21), and we have recently shown that addition of estradiol to "199" medium significantly increases prolactin release by AP explants *in vitro*(22).

These *in vitro* findings can be considered as perhaps the most cogent evidence that prolactin synthesis and release do not depend on hypothalamic or other nervous influences. Other AP hormones show a profound reduction in secretion rate when the pituitary is removed from its normal cranial site and transplanted elsewhere in the body(23). The few *in vitro* studies on AP tissue previously reported indicate that minute amounts of other hormones are produced for a few days only. It remains to be established whether specific factors are elaborated by hypothalamic or other CNS regions which inhibit or otherwise influence prolactin production. Further *in vitro* studies are in progress to determine the direct effects of neurohormones and other agents on prolactin secretion by AP explants.

Summary. Anterior pituitary explants from female immature, mature and postpartum lactating rats were cultured *in vitro* on "199" or Trowell T-8 medium at 35 ± 1°C for periods of 6-14 days. Medium was assayed for prolactin by the intradermal pigeon crop method. Pituitary explants from mature female rats, gassed continuously with 95% O_2 - 5% CO_2 released 5-10 times more prolactin into medium each day than explants exposed only to air atmosphere. The amount of prolactin per AP recovered each day from the culture medium, under continuous gassing, was 2-3 times greater than that present in a fresh AP prior to culture. This proves that prolactin was actively synthesized and released and not merely "leached" out from AP explants. Explants from immature rats elaborated about half as much prolactin per AP per day as explants from mature rats, and explants from lactating rats about twice as much as explants from mature rats. Assays of some explants at the end of culture revealed a low prolactin content, indicating rapid synthesis and release. Histological examination of explants at termination of culture showed that tissue exposed to continuous gassing was almost entirely viable, whereas tissue exposed to air atmosphere had only a thin outer rim of living cells. These results demonstrate that prolactin can be produced in large amounts when AP tissue is cultured *in vitro*.

ADDENDUM

Improvements in our tissue culture techniques have resulted in release into the medium of 10 times as much prolactin per AP per day as was initially introduced into the medium by the fresh tissue. This constitutes dramatic evidence of the ability of the AP to produce prolactin when freed of hypothalamic inhibition.

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Recovery of New Viruses (Coryzavirus) from Cases of Common Cold in Human Adults. (26962)

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Although the common cold continues to be one of the most prevalent of all the illnesses of man, it remains one of the least understood in terms of its etiology. A number of families of viral agents have been shown to be important in causing acute respiratory illnesses of man, especially among children. None of these viruses, however, account for the great mass of acute infections of the upper respiratory tract, especially in adults, which are characterized by a prominent coryza accompanied with mild constitutional symptoms and little or no fever and which are generally classed as the common cold.

What might constitute a breakthrough in the field of the common cold has been reported by Tyrrell *et al.*(1-6). These workers have recorded recovery in primary cell cultures of human fetal kidney and in monkey kidney of a spectrum of viruses which exhibit heterogeneity with respect to cultivability, cytopathogenicity and antigenic composition. In their virus cultivation procedure, Tyrrell *et al.*(1-6) have emphasized the need for maintaining a low pH (0.03% sodium bicarbonate) and low temperature. These agents, collectively called "Salisbury viruses" have been shown to induce cold-like illnesses in human volunteers. Additional viruses were isolated by Hobson *et al.*(7) using similar techniques.

During the past 3 years, our laboratory has been engaged intensively in studies of respiratory diseases of man with special reference to acute respiratory illnesses in children and the common cold in adults. For virus recovery attempts, we chose to employ strains of serially passaged human fibroblastic cells of fetal origin and with diploid karyotype which were recently developed and described by Hayflick *et al.*(8,9) of the Wistar Inst. of Anatomy and Biology.

Through use of such cultures, we have recovered from cases of common cold a group of viruses of small size which are strongly cytopathic for the diploid human fetal lung and kidney cell cultures. These agents are immunologically distinct from the prototype F.E.B. and H.G.P. strains of "Salisbury" viruses and, for purpose of designation, are called coryzavirus because of their association with coryzal disease in man.

The present preliminary report describes the findings to date for 17 coryzaviruses recovered from cases of common cold, and describes the utility of diploid human fetal lung and kidney cells for propagating a variety of viruses. Finally, data are presented which show the need for standard conditions in measuring virus particle size through use of Elford's collodion membranes.

Materials and methods. The patients were employees of Merck & Co. and all but one (M.R.H.) were located in the plant at Rahway, N.J. All were seen by physicians at the plant dispensary. The patients were selected on the basis of having an acute respiratory illness of 4 days or less duration judged on clinical grounds to be of probable viral etiology. Included for control purpose were "normal" individuals who visited the dispensary for some other cause, such as injury. Each patient was examined clinically, was queried concerning recent vaccinations, and the findings were recorded. Two dry cotton swab specimens were collected from the throat. These were extracted immediately into 5 ml of Difco veal infusion broth in a screw cap tube, frozen in dry ice and transmitted to the laboratory where they were thawed, distributed, and stored frozen in dry ice in flame-sealed glass containers until tested up to 1½ years later. A 20 ml sample of blood was taken at the time of the first visit and again 3 to 4 weeks later. The sera were stored frozen at −20°C until tested.

Tissue cultures. Diploid human fetal fibroblast cultures. Strain WI-10 of human fetal kidney and strain WI-26 of human fetal lung were obtained from Dr. L. Hayflick of the Wistar Inst. of Anatomy and Biology(8,9). The cells were propagated in Eagle's medium in Earle's balanced salt solution(10) containing 10% of unheated calf serum. Conventional trypsinization techniques were employed for subculture. During viral propagation, the cultures were maintained in Eagle's medium with 2% of inactivated horse serum or, where noted, in a serum-free medium. The maintenance medium contained 0.006M tris (hydroxymethyl aminomethane) buffer plus 0.09% added sodium bicarbonate. The initial pH was usually around 7.2 to 7.4. *HeLa cell cultures* were grown in Eagle's medium with 10% of inactivated calf serum. During viral growth, the cells were maintained in Eagle's medium with 2% of inactivated horse serum. *Grivet kidney cell cultures.* Trypsinized suspensions of grivet renal cells (*Cercopithecus aethiops*) were planted in Melnick's(11) lactalbumin-yeast extract medium containing 2% of inactivated calf serum. For maintenance purpose, Eagle's medium containing 2% of inactivated chicken serum was used. Penicillin and streptomycin at concentrations of 100 units and 100 μg per ml, respectively, were included in all culture media.

Virus studies. Virus isolation. The throat specimens were diluted 1:2 in Eagle's medium containing 100 units or 100 μg each of penicillin, streptomycin and mycostatin and were spun for 30 minutes at 2000 rpm in the horizontal centrifuge. Supernate was inoculated in 0.25 ml amount, in triplicate, into diploid fetal lung or kidney, HeLa and grivet renal cell cultures. The diploid fetal cultures were incubated at 33°C on the roller drum and the other cell cultures were incubated on the drum at 36°C. All cultures were observed daily for cytopathic effect (CPE) for 10 to 14 days. A single blind passage was made of all cultures which failed to show CPE. Tubes which exhibited CPE were harvested and the contents were stored frozen at —20°C. *Infectivity titrations of Coryzaviruses.* Serial 10-fold dilutions of coryzaviruses recovered in diploid lung or kidney cell cultures were titrated in 0.2 ml amounts in diploid fetal lung cultures containing 2.0 ml of maintenance medium and were incubated on roller drums at 33°C. The titer was the highest initial dilution of virus which caused a CPE by the eighth day post-inoculation. *Serum neutralization test.* Seed stocks of coryzaviruses were prepared in cultures of diploid fetal lung cells. The infected cultures were harvested when CPE was complete (days 3 to 5), were frozen and thawed once, and were clarified by low speed centrifugation. The titers were usually $10^{3.0}$ to $10^{5.0}$ TCD_{50} per 1.0 ml. For neutralization titrations, serial 2-fold dilutions of inactivated serum (56°C, 30 minutes) were incubated for one hour at room temperature with an equal volume of virus diluted to contain 100 TCD_{50} of virus per 0.2 ml. Diploid fetal lung cultures were inoculated in duplicate with 0.2 ml of the serum-virus mixtures and incubated at 33°C on the roller drum. The tests were read on day 3 or 4 of incubation when control cultures showed degeneration of 25 to 50% of the cell sheet. The titer was the highest initial dilution of serum which showed complete suppression of viral CPE.

Biophysical and biochemical procedures. Gradocol membrane filtration. Coryzavirus was propagated in diploid fetal lung cell cultures which were maintained without added serum. The harvested fluid was clarified by low speed centrifugation, 10% of sterile veal infusion broth was added to the supernate, and the mixture was homogenized with 10% of fluorocarbon 113 for 2 minutes at top speed in an Omnimix homogenizer in ice. Final clarification was carried out at 2000 rpm for 10 minutes in the horizontal centrifuge. Aliquots of the material were filtered through Elford's gradocol membranes of various pore size as stated in the text and the filtrates were titrated in quadruplicate in tubes of diploid fetal lung cell cultures. *Sedimentation studies.* Coryzavirus, poliovirus III (Saukett) and adenovirus 2 were spun simultaneously for various time periods at 20°C in the SW-39 head of the model E Spinco ultracentrifuge at an average centrifugal force of 123,600 × g. Following centrifugation, the top 1.0 ml of supernate was carefully sampled and titrated, at 6 tubes per dilution, in the appropriate cell cultures.

The viruses employed in the experiments were propagated in grivet or in diploid fetal lung cell cultures maintained in medium 199 or in Eagle's medium without added serum. Hence, the density and viscosity of the virus-containing fluids were roughly the same. *Electron micrography.* Diploid fetal lung cells infected with coryzavirus were processed according to Palade(12). The cells were embedded in 80% butyl, 20% methyl methacrylate polymerized at 55°C overnight. Sections were cut with a Porter-Blum ultramicrotome equipped with a diamond knife and were examined with a Philips EM-100 electron microscope. *Nucleic acid determination.* The coryzavirus nucleic acid type was determined according to Salzman(13). By this method, tests are made for inhibition of viral synthesis by FUDR (5-fluorodeoxyuridine). For this purpose, diploid fetal lung cell cultures were prepared with Eagle's medium (without serum) containing, in final concentration, 10^{-6}M FUDR, 10^{-5}M FUDR, or 10^{-6}M FUDR plus 10^{-5}M thymidine. Coryzavirus (unknown), vaccinia (known DNA-containing virus) and encephalomyocarditis virus (known RNA-containing virus) were titrated, in quadruplicate, in cultures maintained with each of the above media or with Eagle's medium alone. Determination of nucleic acid type was based on significant suppression of multiplication of DNA virus by FUDR and reversal of this effect by thymidine.

Results. Recovery of coryzaviruses. Throat specimens from 110 patients with mild acute upper respiratory illness and from 45 control cases were specially selected for virus isolation attempts in diploid fetal kidney or lung, in HeLa and in grivet renal cell cultures. The cases were selected on the basis of negative serodiagnostic complement-fixation or hemagglutination-inhibition test findings for influenza A_1, A_2, B, C, parainfluenza 1, 2, 3, adenovirus (group), reovirus (group) and respiratory syncytial virus.

Seventeen throat specimens (15%) from respiratory disease cases and one specimen from a control case yielded cytopathic agents in diploid fetal human cell cultures. These cytopathic effects were first noted between the third and seventh days of cultivation. Subculture did not increase the number of isolates. HeLa cell cultures and grivet renal cell cultures inoculated at the same time with the same throat specimens failed to reveal any cytopathic change indicative of the presence of a virus. Uninoculated or broth inoculated control cell cultures of all types revealed no cytopathic effect. Viruses recovered in the diploid cell and which could not be propagated in HeLa or grivet renal cell cultures were tentatively designated coryzaviruses.

Clinical findings in patients from whom coryzaviruses were recovered. The patients, at time of examination, revealed a clinical picture typical of the common cold. The clinical findings were coryza with nasal edema and discharge. Pharyngitis, which sometimes was painful, and edema of the pharynx were commonly present. About ⅓ of the patients complained of cough or hoarseness. Cervical nodes were present in a small portion of the cases. None of the patients had clinically significant fever and there were no outstanding changes in the total or differential white blood count.

Serologic findings in the patients. The findings in serum neutralization tests with paired sera from 10 cases from whom coryzaviruses were isolated are presented in Table I. The serologic response was generally poor as measured by the neutralization test procedure employed. Only 4 of the 10 patients showed an antibody response against their homologous virus and, in these instances, the convalescent serum titers were only 1:10 or 1:20. No pre-existing homologous antibody was demonstrated in any of the acute phase sera tested. Additionally, as stated above, none of these patients' sera showed diagnostic findings for influenza, parainfluenza, adenovirus, reovirus or respiratory syncytial virus.

Attempts to demonstrate complement-fixing antibody in patients' sera tested with homologous tissue culture coryzavirus fluids gave uniformly negative results.

Biological properties of the coryzaviruses. Cytopathology. All coryzaviruses recovered to date have shown a similar cytopathic effect and this is indistinguishable in diploid fetal kidney and lung cultures. Typical changes

TABLE I. Serum Neutralization Test Findings in Ten Patients from Whom Coryzaviruses Were Recovered.

Case no.	Day of disease	Titer against homologous virus
68	3	<5
	24	20
MRH	2	<5
	27	20
54	1	<2.5
	22	10
211	1	<5
	21	10
1	1	<2.5
	28	<2.5
6	2	<2.5
	25	<2.5
7	2	<2.5
	22	<2.5
53	2	<2.5
	22	<2.5
181	2	<2.5
	25	<2.5
204	1	<2.5
	21	<2.5

in wet and Giemsa stained preparations of diploid lung cell cultures infected with coryzavirus strain 68 are shown in Fig. 1. Cytopathic effect usually began within 2 days post-inoculation and was most often complete within 5 days. Uninoculated diploid fetal cells were typically elongate. Coryzavirus infection caused the cells to separate. Early in the process, a few of the cells assumed a rounded, oval or angular form, became refractile, and some exhibited swelling. This change ultimately occurred throughout the cell sheet. The cells finally were lysed leaving a finally granular debris, naked nuclei, and filamentous structures which might represent disrupted cell membranes. In Giemsa stained preparations, the nuclei showed distortion and wrinkling and stained either more intensely or less intensely than normal cell nuclei. Margination of chromatin and other disruption of nuclei was apparent but no clearly defined inclusion bodies were seen. Cell lysis was clearly shown.

Host range. None of the 10 coryzavirus isolates shown in Table I caused significant cytopathic effect on 2 serial passages in HeLa and grivet renal cell cultures incubated on roller drums at 33°C or at 36°C. No virus could be recovered on subculture into diploid fetal lung cultures of the second passage HeLa and grivet culture material. Groups of one day-old suckling white Swiss mice inoculated intraperitoneally with the 10 coryzavirus strains which titered $10^{3.0}$ to $10^{4.5}$ per ml failed to develop overt signs of illness within 30 days following inoculation. Additionally, the 68 and M.R.H. strains of coryzavirus failed to induce illness within 30 days in 10-12 g mice inoculated intraperitoneally and intracerebrally or in young adult guinea pigs inoculated by the intraperitoneal route. Mice weighing 10-12 g failed to develop illness or grossly detectable lung lesions following intranasal inoculation with these 2 strains. Neither strain 68 nor M.R.H. coryzavirus caused visible pathology on primary passage or on subculture in 7 day-old embryonated chicken eggs inoculated via the yolk sac nor could the virus be recovered in tissue culture from the egg passage material. Embryonated chicken eggs inoculated via the allantoic and amniotic routes and incubated 3 days failed to show pathology or to develop agglutinins for chicken or guinea pig erythrocytes, on primary passage or on subculture, and the virus could not be recovered in tissue culture. None of the strains of coryzavirus showed growth on ordinary bacterial culture media and pleuropneumonia-like organisms could not be cultured from aliquots of strain 68.*

Optimal propagation temperature. No attempt was made to recover coryzaviruses from throat specimens in cell cultures at 36°C rather than at 33°C. It was found, however, that after an initially low titer on first passage at 36°C, strains 68 and M.R.H. of coryzavirus rapidly increased in titer on serial passage giving fluids with infectivity titers as high as were obtained on incubation at 33°C.

Hemagglutination and hemadsorption. None of the 10 agents listed in Table I caused hemagglutination of guinea pig, rooster or type "O" human red blood cells when incubated at room temperature or at 4°C. Additionally, none of the infected dip-

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* These tests were performed by Dr. L. Hayflick.

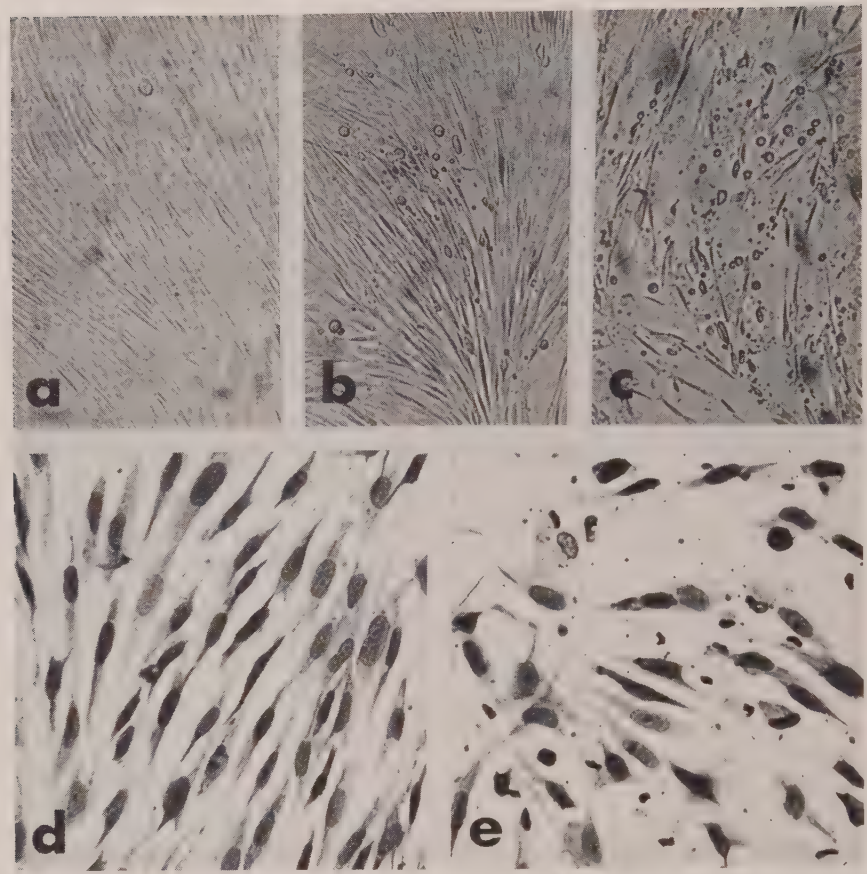

FIG. 1. Cytopathology of coryzavirus group 1 strain 68 in cell cultures of diploid human fetal lung. Wet preparations:
a. Control b. Early CPE c. Advanced CPE × 48 Geimsa stained preparations:
d. Control e. Post infection—24 hours × 170

loid fetal lung cultures showed hemadsorption for guinea pig or rooster erythrocytes at 4°C or at room temperature when tested at a time after cytopathic change had occurred in the cultures.

Antigenic heterogeneity among coryzavi-

TABLE II. Evidence for Serologic Heterogeneity Among Coryzaviruses.

		Coryzavirus isolate									
		Group 1					Group 2	Group 3	Group 4		
Patient's serum	Serum sample	68	54	7	6	53	MRH	211	204	1	181
Group 1											
68	Acute	<5	<5	<5	<5	<5	<5	<5	<5	<2.5	5
	Conv.	*20*	20	10	10	20	<10	<10	<10	<2.5	10
54	Acute	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	2.5	<2.5	<2.5	<2.5
	Conv.	5	*10*	5	10	5	<5	5	<5	<5	<2.5
Group 2											
MRH	Acute	<5	<5	<5	<5	<5	<5	5	<5	<5	<5
	Conv.	<10	<10	<10	<10	<10	*20*	10	<10	<10	<10
Group 3											
211	Acute	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<5	<2.5	<2.5	<2.5
	Conv.	<5	<5	<5	<5	<5	<2.5	*10*	<5	<5	<5

ruses. Paired sera from cases 68, 54, M.R.H. and 211, which showed a neutralizing antibody increase, were titrated with the 10 isolates presented in Table I. The findings are summarized in Table II. The viruses were separated into 4 distinct groups designated 1, 2, 3 and 4. Group 1 strains 68, 54, 7, 6 and 53 appeared to be alike antigenically. Group 2 and 3 strains M.R.H. and 211 appeared to be represented by only a single example. No titer increases were shown against group 4 strains so that degree of homogeneity could not be measured.

Lack of antigenic relationship of coryzaviruses to known viruses. Antisera† against the prototype F.E.B. and H.G.P. strains of "Salisbury" viruses failed to neutralize any of the 10 isolates shown in Tables I and II when used at serum dilutions which completely neutralized the homologous agents. Additionally, the F.E.B. and H.G.P.† viruses were not neutralized by groups 1, 2 or 3 sera from patients 68, 54, M.R.H. and 211. None of the 10 coryzavirus isolates were neutralized by hyperimmune animal antisera of known potency against ECHO virus types 11, 20, 22 or 28 or by Coe (Coxsackie A 21) (14) or Pett(15) strains of virus. Finally, neither coryzavirus strain 68 nor M.R.H. was neutralized by potent antisera against the remainder of the presently recognized ECHO virus types.

TABLE III. Gradocol Membrane Filtration of Strain 68 Coryzavirus.

Avg. pore dia. of membrane, mμ	Sample tested		Result	
			CPE	TCD_{50}/ml
Exp. 1		ml		
320	1st	10	neg.	
220	1st	10	"	
100	1st	10	"	
	2nd	10	pos.	
85	1st	10	neg.	
	2nd	10	pos.	
61	1st	10	neg.	
	2nd	10	pos.*	
	3rd	10	"	
40	1st	10	neg.	
	2nd	10	pos.*	
	3rd	10	"	
33	1st	10	neg.	
	2nd	10	"	
	3rd	10	"	
	4th	10	pos.*	
Exp. 2				
61	1st	30		$10^{4.0}$
	2nd	20		$10^{5.2}$
40	1st	30		$10^{2.2}$
	2nd	20		$10^{3.2}$
33	1st	30		$10^{1.2}$
	2nd	20		$10^{3.2}$
27	1st	30		neg.
	2nd	20		Trace (3/6 tubes pos.)

* Appearance of CPE delayed 3 days.

Biophysical and biochemical properties of coryzaviruses. Gradocol membrane filtration. Strain 68 of coryzavirus prepared in diploid fetal lung was used. This material was largely freed of extraneous proteinaceous material by centrifugation and by fluorocarbon treatment. Initial experiments, in which 10 ml volumes of virus were filtered, showed passage of the virus through 760 mμ but not 320 or 220 mμ average pore diameter (APD) membranes. Early centrifugation data suggested a smaller particle size than indicated by filtration and it appeared likely, therefore, that the virus was being adsorbed to the filters. To overcome this, larger quantities of virus-containing fluid were passed. The importance of volume of virus filtered is clearly shown in Table III. In Exp. 1, based on a 10 ml filtration sample, the virus failed to pass even a 320 mμ APD membrane. Membranes of 100, 85, 61 and 40 mμ APD required the filtration of 20 ml of fluid before virus could be detected in the filtrate, and the 33 mμ membrane required 30 ml of fluid. These relationships are exemplified further in Exp. 2 in which infectivity titrations were carried out. It is seen that a small amount of virus was detected after filtration through the 27 mμ APD membrane. Considering that only a trace of virus was detected in the 27 mμ filtrate and applying Black's factor of 0.64(16) to convert pore diameter to virus particle size, the virus diameter is estimated to be about 17 mμ.

Centrifugation.‡ A second measure of

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† These viruses and antisera were kindly furnished by Dr. D. A. J. Tyrrell.

‡ The authors are indebted to Dr. A. A. Tytell and to Mr. D. S. Spicer for assistance in performing the centrifugation runs.

TABLE IV. Comparison of Sedimentation of Coryzavirus 68, Poliovirus III and Adenovirus 2 at 123,600 × g, According to Time.

Virus*	Log₁₀ per ml reduction in TCD₅₀ when centrifuged for time periods 0	15 min.	30 min.	45 min.	60 min.
Coryzavirus—68	0	$10^{0.3}$	$10^{0.2}$	$10^{0.4}$	$10^{1.4}$
Poliovirus III (30 mμ dia.)	0	$10^{0.4}$	0	$10^{1.5}$	$10^{2.2}$
Adenovirus 2 (70 mμ dia.)	0	$10^{1.8}$	$10^{1.4}$	$10^{2.9}$	$10^{2.9}$

* Initial virus titers, Coryzavirus 68, $10^{4.5}$ TCD_{50}/ml; poliovirus III, $10^{6.9}$; adenovirus 2, $10^{7.1}$.

particle size of strain 68 coryzavirus was achieved by comparing the sedimentation rate of this virus with that of poliovirus III, with known particle size of about 30 mμ, and that of type 2 adenovirus, with known particle size of about 70 mμ. The experiments were conducted as described in the materials and methods and the findings (Table IV) are expressed in terms of reduction in infectivity in the top 1 ml of supernate, according to length of centrifugation period. It is seen that more than 90% of the adenovirus was removed from the upper 1 ml of supernate within 15 minutes. Forty-five minutes were required to accomplish this for poliovirus and 60 minutes for coryzavirus. Assuming similar density, this places the size of coryzavirus at something less than poliovirus.

Electron micrography§. Thin sections were prepared of diploid fetal lung cultures infected with coryzavirus 68 and of uninfected cultures. An electron micrograph of an infected cell is shown in Fig. 2. Spherical structures about 18 mμ in diameter were present in a crystalline array. No such structures were seen in uninoculated cells. These particles are tentatively ascribed to coryzavirus.

Nucleic acid typing. The findings of the experiments conducted according to Salzman (13) are shown in Table V. Coryzavirus 68 was not suppressed by FUDR and hence is classed as an RNA virus. Vaccinia virus, known to be of DNA type, was inhibited by FUDR but this was reversed by added thymidine. Encephalomyocarditis virus, known

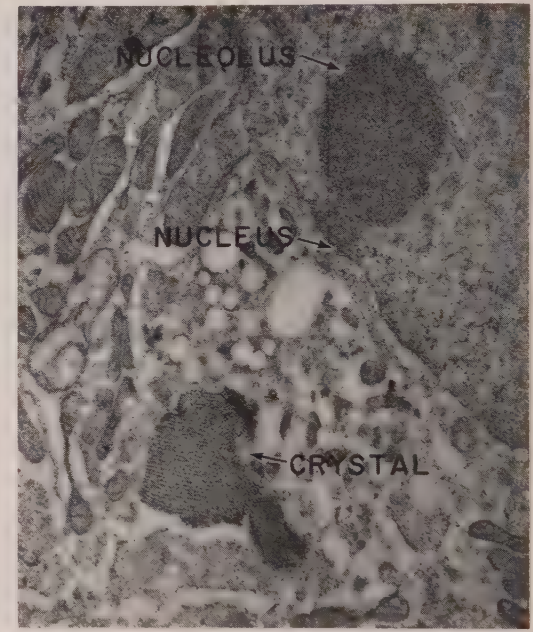

FIG. 2. Electron micrograph of a thin section of a diploid cell infected with coryzavirus group 1 strain 68. × 17,400.

TABLE V. Determination of Nucleic Acid Type of Coryzavirus 68 Through Use of FUDR.**

Virus	Additive to culture medium None	FUDR 10^{-6}M	FUDR 10^{-5}M	FUDR 10^{-6}M plus thymidine 10^{-5}M	Virus nucleic acid type
Coryzavirus 68	$10^{3.4}$*	$10^{3.7}$	$10^{3.4}$	$10^{3.4}$	RNA
Encephalomyocarditis	$10^{7.4}$	$10^{7.4}$	$10^{7.7}$	—	RNA
Vaccinia	$10^{6.7}$	$10^{3.2}$	—	$10^{6.7}$	DNA

* Infectivity titers expressed as TCD_{50} per ml.
** 5-fluorodeoxyuridine.

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§ The authors are indebted to Dr. A. I. Schepartz and to Mr. R. W. Ziegler for preparing the sections and for the electron micrography.

TABLE VI. Summary of Presently Known Biological and Physical Properties of Coryzavirus.

A. Clinical
  1. Recovered from patients with common cold.
  2. Patients develop antibody in response to infection.

B. Biological properties
  1. Grow and produce CPE in diploid human fetal kidney and lung cell strains.
  2. Neither propagate nor produce significant CPE in HeLa or in grivet renal cell cultures.
  3. Readily recovered in diploid human fetal cell cultures incubated at 33 ° C; also propagate at 36 ° C.
  4. Cytopathic effect is primarily cell rounding, swelling, lysis. No inclusion bodies seen.
  5. Fails to grow or cause detectable damage in suckling or adult mice, guinea pigs, or embryonated chicken eggs by usual routes of inoculation.
  6. Fails to produce hemagglutinins and fails to cause hemadsorption.
  7. Antigenically heterogeneous; at least 4 groups recognized (groups 1, 2, 3 and 4).
  8. Serologically unrelated to "Salisbury" prototypes FEB and HGP; unrelated to presently recognized ECHO, Coe, Pett and Reoviruses.
  9. Fails to propagate on ordinary culture media, including media for pleuropneumonia.

C. Biophysical and biochemical properties
  1. About 17 to 18 mμ as estimated from gradocol membrane filtration, centrifugation and electron micrography data.
  2. Resistant to ether and to fluorocarbon 113.
  3. RNA virus as determined by lack of FUDR inhibition.

to be of RNA type, was not affected by FUDR.

Ether resistance. Coryzavirus 68, prepared with serum-free medium and titering $10^{4.2}$ TCD_{50} per ml, was treated with 20% diethyl ether. There was no loss of infectivity titer on storage overnight at 4°C.

Summary of known properties of coryzavirus. Table VI summarizes the known biological, biophysical and biochemical properties of coryzavirus. Most of the data were collected using strains 68 and M.R.H. of coryzavirus.

Viral susceptibility spectrum of diploid fetal cell cultures. Diploid fetal lung and kidney cell cultures appeared equally sensitive to coryzaviruses and equally suitable for work with these agents. In studies conducted in collaboration with Dr. L. Hayflick, the susceptibility of diploid fetal kidney strain WI-10 to a variety of viral agents was measured. The findings are presented in Table VII.

In virus recovery attempts from human respiratory specimens of known content, the fetal kidney failed to detect parainfluenza 2, respiratory syncytial or adenovirus 5 viruses. By contrast, these were detected in cultures of HeLa or grivet renal kidney or both. This indicates a lesser sensitivity for detection even though these same viruses with the exception of parainfluenza 2, appear to propa-

TABLE VII. Susceptibility of Diploid Human Fetal Kidney Cell Cultures (Strain WI-10) to a Variety of Viral Agents as Measured by Development of Cytopathology or of Hemadsorption Effect.

Virus	Effect	Virus	Effect
Enterovirus		Coryzavirus	yes
Poliovirus I (Mahoney)*	yes	Reovirus 1	"
I (Chat)*	"	Myxovirus	
II (TN)*	"	parainfluenza 1	no
III (Fox)*	"	2	"
Coxsackie A_1*	no	3	"
A_9	yes	Adenovirus 2*	"
A_{13}*	"	12*	"
A_{21} (Coe)	"	Respiratory syncytial	"
B_1*	no	Measles*	"
ECHO 9*	yes	Varicella*	"
11	"	Vaccinia*	"
20	"	Arborvirus	
21*	"	WEE*	"
22	"	Yellow fever (17D)*	yes
28	"	Encephalomyocarditis	
Pett	"	EMC*	yes
Salisbury viruses		Mengo*	"
FEB	yes	Rabies*	yes (Partial)
HGP	"	Polyoma	no

* Results obtained by Dr. Hayflick. Presented by permission.

gate in the fetal kidney cells. It would appear, therefore, that the diploid fetal cells should not be used alone for diagnostic test purpose.

Discussion. The findings presented reveal that viruses have been recovered from cases of common cold in the human adult. This illness has been outstanding in the past for its failure to yield to studies of its etiology. Recent studies, not reported herein, have shown recovery also of similar viruses from children with respiratory illness. Recovery of the new viruses was made possible by the use of newly developed cell cultures derived from human fetal kidney and lung and maintained in serial passage in a diploid state(8,9). Because of their association with illness of predominantly coryzal nature, these agents are called coryzaviruses.

The extent to which coryzaviruses are responsible for or are involved in cases of common cold cannot be stated from the present data. The recovery of coryzavirus from 15% of 110 cases of common cold in contrast to only one of 45 (2%) "normal" persons, together with the demonstration of development of antibody in a portion of cases during convalescence, provides evidence for the etiologic role of these agents in the common cold. Failure to recover virus or to demonstrate antibody increase against other known respiratory viruses lends additional support for the primary role of coryzavirus in the etiology of cases studied.

The weak antibody response or the failure to respond serologically to homologous coryzavirus, among the cases studied, might be due to a relative insensitivity of the neutralization technique employed to detect antibody. Alternatively, it might reflect a poor antibody response to a possibly superficial infection with coryzavirus and may, in turn, be responsible in part at least for the apparent transient immunity in certain common colds.

The coryzaviruses appear to be unique in their limited host range, causing CPE in diploid fetal cells but failing to propagate or show demonstrable effect in HeLa and grivet kidney cells as well as in a variety of common laboratory hosts. The size of the virus appears to be very small. Coryzavirus shares with many other agents, *e.g.*, enteroviruses, the property of ether resistance and RNA. The agent has failed, to date, to show hemagglutination or hemadsorption.

The possible relationship of the coryzaviruses to certain of the "spectrum" of "Salisbury" viruses cannot be ascertained with certainty at the present time. The coryzaviruses differ immunologically from the prototype F.E.B. and H.G.P. Salisbury agents and are readily distinguished from H.G.P. prototype by failure to grow and produce CPE in grivet kidney cell cultures. Tyrrell *et al.* (1-6) emphasize the importance of reduced bicarbonate and lowered temperature for growth of the Salisbury strains. Ordinary bicarbonate concentrations, used in conjunction with tris buffer, were employed for isolation and propagation of the coryzaviruses. Although propagated routinely at 33°C, coryzavirus was readily adapted to multiplication at 36°C. Coryzavirus, like the Salisbury viruses, was ether and fluorocarbon resistant. The H.G.P. prototype of Salisbury virus was held back by a 69 $m\mu$ APD collodion filter(3).

In the present studies, a minimal detectable amount of coryzavirus strain 68 passed through a 27 $m\mu$ APD Elford collodion filter, and this became apparent only after 30 ml had already been filtered through the membrane. Determination of particle size by collodion membrane filtration is clearly influenced by several vagaries, most important of which are the relative purity of the material being filtered, concentration of virus in the fluid, and volume of fluid passed. Applying Black's factor of 0.64(16), the size of coryzavirus 68 is estimated to be about 17 $m\mu$ diameter. This is in good agreement with the electron microscope and centrifugation data.

The propagation in human diploid fetal cells of coryzaviruses raises the question of vaccines. Utilizing frozen cell banks, the diploid cell provides a potential for almost unlimited quantities of tissue culture material. The retention by such cells of a diploid karyotype and of the other properties considered characteristic of "normal" cells (9), answers most of the objections raised in the past to employment of serially passaged cell cultures for human vaccine purpose. The degree of diversity of antigenic types in the coryzavirus group and the extent to which these agents contribute to the common cold must be the subject of further research. The poor antibody response to the natural infection with coryzavirus should encourage the use of a killed vaccine in which an adequate immunizing dose of antigen can be applied.

Summary. Viruses have been recovered in diploid cell cultures of human fetal lung or kidney from cases of common cold in human adults and children. These agents, which comprise at least 4 serologic groups, appear to be related etiologically to the common cold in man. Because of their association with coryzal disease and for purpose of designation, the agents are called coryzavirus. Coryzavirus has failed to propagate in HeLa or grivet renal cell cultures, did not produce pathology in common laboratory hosts, and appears to be unrelated to the known respiratory viruses of man. Similarities to and differences from the "spectrum" of "Salisbury" agents are discussed. The agent appears to be of very small particle size and is estimated to be about 17 to 18 mμ in diameter. Other biological, biophysical and biochemical properties of coryzavirus are presented.

ADDENDUM

A number of strains of coryzavirus were recovered from cases of colds both in adults and in children. Cross-neutralization tests with animal antisera have revealed at least 6 distinct serotypes of coryzavirus. Only one-third of strains recovered to date fall into these 6 types. The low incubation temperature (33°C) initially used to recover virus in the diploid cells was found unnecessary for at least 3 of the serotypes and routine 36°C temperature of incubation was often employed in the later work.

The final taxonomic position of the coryzaviruses must await further study. The agents herein designated coryzavirus are similar to the enteroviruses with respect to size, ether resistance, and RNA content and may fall within the general enterovirus group as presently defined. The possible relationship of the coryzaviruses to certain of the Salisbury agents cannot be decided until further cross-serologic tests are made and until precise and comparable biophysical test results are available. The degree of homogeneity among both groups of agents has not been defined.

Andrewes *et al.* (*Virology,* 1961, v15, 52) in a recent publication have proposed to call the Salisbury cold viruses rhinoviruses and to place them in a suggested family of "Nani-viruses" which would include the present enteroviruses, foot and mouth disease virus, Teschen disease, encephalomyocarditis and a number of other viruses. Johnson and associates (unpublished) have recently recovered agents from cases of common cold in primary human cell cultures and in the diploid human fetal cell cultures employed in the present investigations. These workers have recognized at least 6 distinct serotypes and have proposed that their viruses be included as types of enterovirus.

We are indebted to Drs. S. M. Hoch, W. Freeborn and H. H. Palmer for collection of specimens and for clinical observations. Gratitude is also expressed to B. Baron, M. Salvatore, I. Weinberger, and R. Roehm for technical assistance.

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Interaction of Fragment III of Rabbit Gamma Globulin and Guinea Pig Complement.* (26963)

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Porter(1) has reported that rabbit antibody, when treated with crystalline papain, is degraded into 3 fragments. Fragments I and II are univalent and capable of combining specifically with antigen, while fragment III retains the antigenic properties of gamma globulin. Recently we examined the immunochemical properties of the fragments of rabbit γ_2 hemolytic and agglutinating antibodies resulting from digestion with papain (2). We found that all 3 fragments inhibited immune hemolysis. The inhibitory property of III is nonspecific, since III derived from rabbit antipertussis gamma globulin also inhibited immune hemolysis.

In view of the earlier assumptions of Heidelberger and co-workers(3) that complement (C′) yields easily dissociable combinations with dissolved antibody and firm combinations with certain immune aggregates, the possible role of fragments of gamma globulin in both combinations was investigated. Rabbit antibody can be degraded also with pepsin producing a bivalent fragment which is converted to univalent fragments when a disulfide splitting reagent is added(4). This work is, therefore, concerned with the study of additional properties of fragment III and of the fragments obtained by degradation of normal and immune rabbit gamma globulins with pepsin.

Material and methods. Antisera to sheep erythrocytes were prepared by intravenous injection of rabbits with 1.0 ml of a suspension of boiled sheep red cell stromata in saline (1 mg N/ml) thrice weekly for 4 weeks. Rabbits were bled 7 days after the last injection and sera were pooled. Rabbit antipertussis serum was kindly supplied by Miss Jessie L. Hendry of this Division.

Gamma globulin fractions were obtained by fractionation of normal or immune rabbit sera by the Na_2SO_4 precipitation technic of Kekwick(5) and the modified procedure(6) of anion exchange chromatography(7). Gamma globulins were degraded with crystalline papain (Lot 5503, Worthington Biochemical Corp., Freehold, N. J.) and the fragments were isolated by the methods outlined by Porter(1). Digestion of gamma globulin with pepsin (Lot 643, Worthington Biochemical Corp.) was carried out according to the method of Nisonoff and coworkers(4).

Sheep erythrocyte suspensions (E) were prepared as described by Mayer *et al.*(8), except that the concentration of cells was 2×10^9 cells/ml. Veronal-saline buffer(8) containing 1.5×10^{-4} M Ca^{++} and 5.0×10^{-4} M Mg^{++} was used for all dilutions and washings. Pooled guinea pig sera were the source of complement (gp C′) and were stored in small portions at $-30°C$.

Titrations of hemolysin activity of rabbit antisheep erythrocyte serum or antiserum fractions were carried out essentially as outlined by Kabat and Mayer(9). Each reaction tube contained 0.2 ml of E, 0.4 ml of gp C′ (at a concentration which contained four 50% hemolytic units when added to a comparable number of optimally sensitized cells), and 0.2 or 0.4 ml of antiserum or fraction at varying dilutions. Buffer was added to give a final volume of 1.6 ml. The tubes were incubated for 60 minutes at 37°C, centrifuged, and the supernatant fluid, after being diluted 1:4 with distilled water, was read at 541 mμ in Beckman DU spectrophotometer. Hemolytic activity is expressed as number of 50% hemolytic units (H_{50})/ml undiluted fraction. Agglutinin titers were determined in the manner described by Kabat and Mayer(9). Aliquots (0.2 ml) of antiserum or fractions at various dilutions were added to 0.1 ml of E in a final volume of 0.8

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* This study was supported by research grant E-3757 from the National Institute of Allergy and Infectious Diseases.

ml. Tubes were incubated for 60 minutes at 37°C and read. Agglutinin unit is expressed as the highest dilution of serum giving definite agglutination. Agglutinin activity is the number of units/ml undiluted fraction. Inhibition of immune hemolysis by fragments of gamma globulin was tested by the same procedure except that fragments were allowed to incubate with E and buffer for 30 minutes at 0°C prior to addition of γ_2 hemolytic antibody and gp C′.

The method of quantitative complement fixation by immune aggregates is that outlined by Kabat and Mayer(9).

Results. Preincubation of fragment III with reactants of immune hemolysis. Varying amounts of normal rabbit gamma globulin and fragment III, derived from the former by papain digestion, were preincubated with either E or gp C′ for 30 minutes at 0°C. The remaining reactants (gp C′ or E and γ_2 hemolytic antibody) were added and tubes were incubated at 37°C. In addition, tubes containing varying amounts of gamma globulin or fragment III were allowed to react with E, γ_2 hemolytic antibody, and gp C′ with no preincubation period. The results are shown in Fig. 1. Preincubation of gamma globulin or fragment III with gp C′ results in greatest inhibition of immune hemolysis. There is little or no change in inhibitory property of gamma globulin or fragment III when preincubated with E as compared with no preincubation.

Interaction of fragment III with E. Varying amounts of fragment III derived from a gamma globulin fraction of rabbit antisheep erythrocyte serum were preincubated with E for 30 minutes at 0°C. Tubes were centrifuged and the residual cells were washed twice with 1.0 ml buffer and reacted with γ_2 hemolytic antibody and gp C′. Each supernatant fluid was added to fresh E. γ_2 hemolytic antibody and gp C′ were then added, and tubes were incubated at 37°C. Similar amounts of fragment III were reacted with E, γ_2 hemolytic antibody, and gp C′. Though most of inhibitory property of fragment III resides in the supernatant fluid, there is some interaction of fragment III, at high concentrations, with E (Fig. 2).

Effect of pepsin-digested immune and normal gamma globulins on immune hemolysis. Normal and immune (antipertussis and antisheep erythrocyte) rabbit gamma globulins were degraded with pepsin and resulting fragments were examined for inhibitory properties in immune hemolysis. In addition, fragment III derived from papain digest of rabbit antipertussis gamma globulin was tested. Fig. 3 indicates that fragments of peptic digestion of normal and antipertussis gamma globulins do not affect the immune hemolytic reaction, while comparable concentrations of a similar fragment derived from antisheep erythrocyte gamma globulin and fragment III of antipertussis gamma globulin are inhibitory.

TABLE I. Effect of Pepsin on Activities of Rabbit Antisheep Erythrocyte Gamma Globulin.

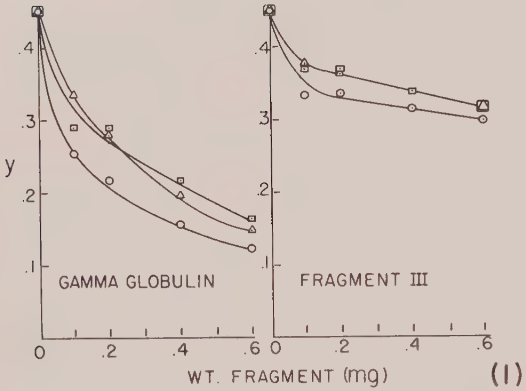
	Agglutinin activity	Hemolytic activity
Rabbit antisheep erythrocyte gamma globulin	17	12,000
Pepsin digest	17*	<100

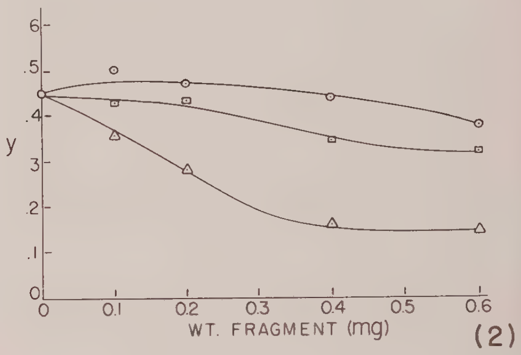
* After 16 hr at room temperature. No activity after 60 minutes at 37°C.

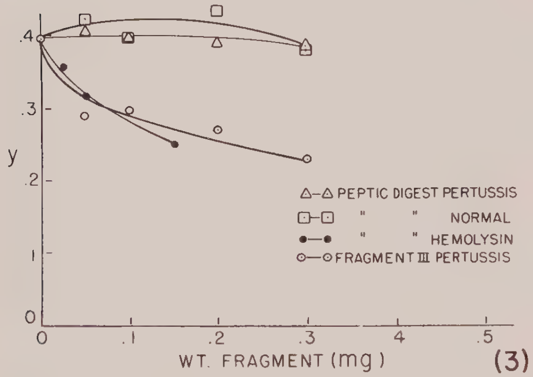
The pepsin digest of rabbit antisheep erythrocyte gamma globulin was tested for agglutinin and hemolytic activity. The results are shown in Table I. It is of interest to note that though hemolytic activity is lost, agglutinating ability is retained, albeit at a slower rate.

In view of these results, the ability of the pepsin digest of rabbit antisheep erythrocyte gamma globulin to fix complement was examined. One milliliter of a solution of rabbit antisheep erythrocyte gamma globulin (26 μg N/ml) and 1.0 ml of pepsin digest (18 μg N/ml) were reacted with varying amounts of boiled sheep erythrocyte stromata in the presence of 110 $C'H_{50}$. The fixation of gp C′ by these systems is depicted in Fig. 4. Treatment of rabbit antisheep erythrocyte gamma globulin with pepsin results in about 70% less fixation of gp C′.

Discussion. It was reported earlier(2) that nonspecific inhibition of immune hemolysis by fragment III of rabbit gamma globulin might be due to a site on the fragment which combines with the red cell similar to the skin attachment site(10) and mem-







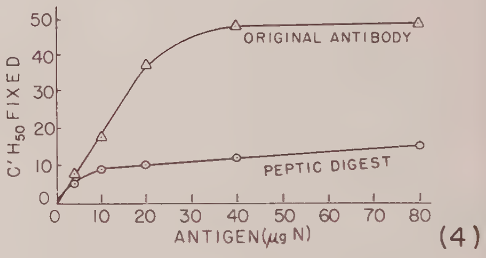


FIG. 1. Effect of preincubation of gamma globulin or fragment III with reactants of immune hemolysis

⊙—⊙ Preincubation with gp C′
□—□ Preincubation with E
△—△ No preincubation

FIG. 2. Interaction of fragment III of rabbit anti-sheep erythrocyte gamma globulin with E

△—△ Inhibition of immune hemolysis by fragment III
⊙—⊙ Inhibition of immune hemolysis by pretreatment of E with fragment III
□—□ Inhibition of immune hemolysis by residual fragment III

FIG. 3. Effect of peptic digests of normal and immune rabbit gamma globulin on immune hemolysis

FIG. 4. Fixation of gp C′ by rabbit antisheep erythrocyte gamma globulin and its peptic digest

Intact gamma globulin, 26 μg N
Peptic digest, 18 μg N

brane transmission site(11) associated with this fragment. There appears to be some interaction of the fragment with the red cell (Fig. 2). Inhibition, however, is due primarily to the ability of the fragment to associate with gp C′. Evidence for this is the increased inhibition upon preincubation of fragment III (or gamma globulin) with gp C′ (Fig. 1) and the loss of inhibitory properties of normal and antipertussis gamma globulins upon treatment with pepsin (Fig. 3). Peptic digestion of rabbit gamma globulin decreases the sedimentation constant by removing an inert portion of the molecule and leaves a bivalent fragment that appears to be composed of Porter's fragments I and II(4). The inhibition of immune hemolysis by the pepsin digest of rabbit antisheep erythrocyte gamma globulin is specific since this digest is capable of agglutinating sheep erythrocytes but it is not capable of lysing these erythrocytes in the presence of gp C′. This inability to lyse sheep red cells is probably due to loss in ability to fix gp C′. It appears therefore that the portion of rabbit gamma globulin that combines with gp C′(3) resides in fragment III. The decrease in rate of agglutination by treatment of rabbit antisheep erythrocyte gamma globulin with pepsin implicates to some extent the role of fragment III in another immune phenomenon.

Summary. Fragment III derived from papain digestion of rabbit gamma globulin inhibits immune hemolysis primarily by its ability to interact with gp C′ and to some extent by interacting with sheep erythrocytes. Peptic digests of normal and antipertussis rabbit gamma globulins do not inhibit immune hemolysis. Peptic digests of rabbit antisheep erythrocyte gamma globulins are capable of agglutinating sheep erythrocytes at a slower rate when compared to that of the intact antibodies. The latter digests will not lyse sheep erythrocytes in the presence of gp C′, because they fix less complement than intact antibody.

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Effects of Psychotropic Compounds on Enzyme Systems, I. Inhibition of Human Plasma and Erythrocyte Cholinesterases.* (26964)

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Although there is some literature on enzyme inhibition by psychotropic compounds, good quantitative data, particularly relating *in vivo* with *in vitro* effects in the same species are relatively rare. Some enzyme systems, for example choline acetylase, have been largely ignored, probably because of difficulty of assay rather than lack of significance of this enzyme. We propose to report investigations on *in vivo* and *in vitro* quantitative inhibitory effects of a large number of compounds with psychotropic activity on various enzyme systems and to attempt to correlate psychotropic action with effects on enzyme systems.

Most of the published inhibition studies have been either in qualitative terms ("inhibits", "enhances") or in semi-quantitative terms (50% inhibition = I_{50}). The I_{50} value, however, is dependent upon substrate concentration, so that it is frequently difficult to compare numerical values obtained by different assay methods. Moreover, determination of I_{50} values does not necessarily entail an analysis of the kinetics of inhibition. It has therefore been found desirable to determine K_i values (K_i = inhibitor constant), as defined by Dixon and Webb(1).

Tranquilizers of the phenothiazine type have been found to be *in vitro* inhibitors of cholinesterases(2,3). It has also been suggested(2) that the effectiveness of phenothiazine tranquilizers might be related to their cholinesterase inhibitory activity.

Material and methods. The tranquilizers

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[†] Some of this work carried out in partial fulfillment of requirements for M. S. degree, New Mexico Highlands Univ.

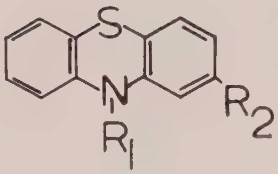
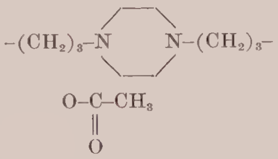
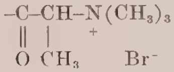


FIG. 1. Phenothiazines used.

Compound	R_1	R_2
Promazine	$-(CH_2)_3-N(CH_3)_2$	–H
Chlorpromazine	$-(CH_2)_3-N(CH_3)_2$	–Cl
Triflupromazine	$-(CH_2)_3-N(CH_3)_2$	$-CF_3$
Thiopropazate		–Cl
Secergan	 $-\underset{\underset{O}{\Vert}}{C}-\underset{\underset{CH_3}{\vert}}{CH}-\overset{+}{N}(CH_3)_3 \quad Br^-$	–H

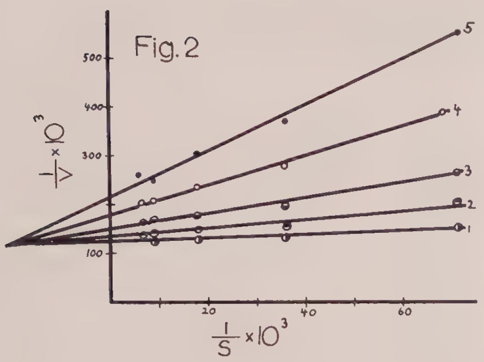
used in these experiments were kindly supplied as follows: chlorpromazine, Smith Kline and French Labs; promazine, Wyeth Labs; secergan, Astra Pharmaceutical Products, Inc.; thiopropazate, G. D. Searle and Co.; triflupromazine, The Squibb Inst. for Medical Research. (The structures of these compounds are given in Fig. 1.) Two of the substrates used, acetyl-β-methyl-choline chloride (MeCh) and acetylcholine iodide (AcCh), were commercial preparations. The third substrate, butyrylcholine iodide (BuCh), was prepared in this laboratory according to the method of Tammelin(4). The human blood used, obtained from healthy adult volunteers, was collected directly into chilled flasks containing ethylene diamine tetraacetic acid. It was centrifuged immediately after collection at 3,000 rpm for 10 minutes at 4°C. Plasma was diluted to twice its original volume with Krebs Ringer solution and served as the source of plasma cholinesterase (ChE). Erythrocytes were washed twice with isotonic saline then diluted to 3 times the original volume with 0.001% saponin solution and served as the source of erythrocyte acetylcholinesterase (AcChE). Both plasma and the erythrocyte preparations were stored at 4°C, and were used without further purification. No loss in activity was observed over a period of 10 days.

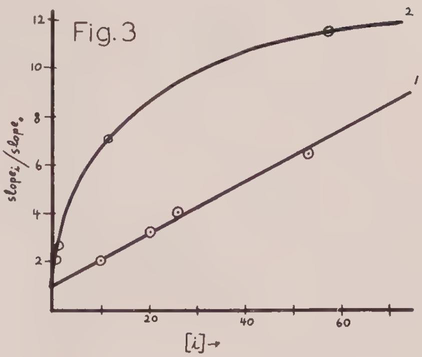
Cholinesterase activities were measured by the manometric method of Ammon(5) at pH 7.4, 37°C, using 5% CO_2-95% N_2 as the gas phase. All inhibitors were dissolved in Krebs Ringer solution immediately before use, and were incubated with the enzyme preparation for 40 minutes before addition of substrates. Manometers were read every 5 minutes for a total of 30 minutes. All manometer readings were converted to μl CO_2 and all data expressed as velocity $= \mu l\ CO_2/min$. All data given are averages of at least 3 different runs, each in duplicate.

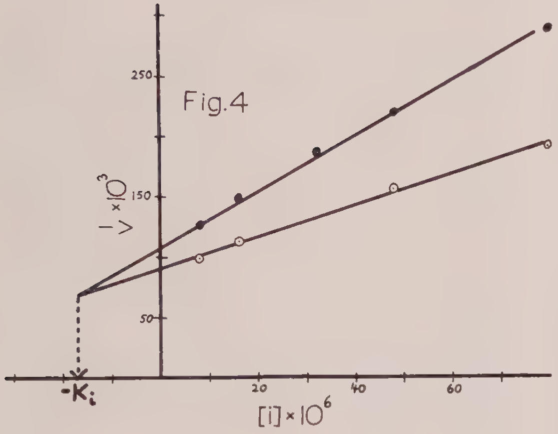
Inhibitor constants were calculated either by a direct graphical method, plotting $1/V$ vs. [i] for at least 2 different substrate concentrations, or by plotting $1/V$ vs. $1/S$ for control and inhibited runs. In the latter method, the ratios of the slopes of the inhibited and control lines were used to calculate K_i. For purely competitive inhibition, $\frac{\text{slope inhibited line } (s_i)}{\text{slope control line } (s_o)}$ plotted vs. [i] gives a straight line. For partially competitive inhibition, $\frac{s_i}{s_o} = \frac{1 + i/K_i}{1 + (i/K_i)(K_m/K_{m'})}$; a plot of the ratios of the slopes of the lines vs. [i] will not yield a straight line, and therefore several inhibitor concentrations are needed to calculate K_i. When the lines obtained in a plot of $1/V$ vs. $1/S$ for different inhibitor concentrations do not meet on either the $1/V$ or the $1/S$ axis, inhibition is of the mixed type and K_i can be calculated from the ratios of the slopes, as in the case of competitive inhibition.

Results. Erythrocyte AcChE with MeCh as substrate was inhibited to varying extents by the 5 phenothiazines used, with K_i ranging from 1.5×10^{-4} M to 1.2×10^{-5} M (Table 1). Although the differences in K_i values were considerable, all followed the mixed type kinetics as is illustrated graphically for thiopropazate (Fig. 2).

The inhibition of plasma ChE with AcCh as substrate was generally more pronounced than the inhibition of AcChE. Competitive kinetics were found with secergan, promazine, chlorpromazine and triflupromazine, while







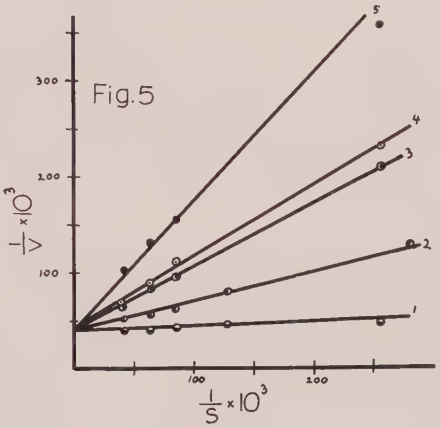


FIG. 2. Inhibition of AcChE by thiopropazate, MeCh as substrate
1. No inhibitor
2. Inhibitor conc. 1.14×10^{-3} M
3. " " 3.46×10^{-3} M
4. " " 6.96×10^{-3} M
5. " " 10.4×10^{-3} M

FIG. 3. Ratios of slopes for chlorpromazine and for thiopropazate

FIG. 4. Inhibition of ChE by secergan, AcCh as substrate
1. $S = 8.0 \times 10^{-3}$ M
2. $S = 16 \times 10^{-3}$ M

FIG. 5. Inhibition of ChE by promazine, BuCh as substrate
1. No inhibitor
2. Inhibitor conc. 1.2×10^{-5} M
3. " " 3.1×10^{-5} M
4. " " 3.8×10^{-5} M
5. " " 7.5×10^{-5} M

thiopropazate exhibited the partially competitive type of reaction (Fig. 3). A Dixon and Webb type plot is shown for secergan in Fig. 4. When BuCh was used as substrate instead of AcCh, similar results were obtained except in the case of secergan, where the competitive inhibition with BuCh was considerably greater than with AcCh. The competitive inhibition with BuCh as substrate is shown graphically for promazine in Fig. 5.

Discussion. Four of the phenothiazines used are tranquilizers, whereas the fifth, secergan, is primarily an antispasmodic(6). Secergan was included in this study since it was expected that it would be a stronger ChE inhibitor than the other phenothiazines because of its quaternary ammonium nitrogen. This was not found to be true in the case of the ChE-AcCh system. Possibly, the close proximity of the carbonyl group to the charged

TABLE I. K_i Values and Type of Inhibition.

Compound	Rec. dose (mg)	AcChE-MeCh		ChE-AcCh		ChE-BuCh	
		K_i	Type	K_i	Type	K_i	Type
Thiopropazate	15	$(9.8 \pm .6) \times 10^{-4}$	mixed	$(6.7 \pm .3) \times 10^{-5}$	partially competitive	$(4.4 \pm .3) \times 10^{-5}$	partially competitive
Triflupromazine	50	$(5.1 \pm .1) \times 10^{-5}$	"	$(3.5 \pm .4) \times 10^{-5}$	competitive	$(2.5 \pm .2) \times 10^{-5}$	competitive
Chlorpromazine	10-100	$(7.0 \pm .7) \times 10^{-5}$	"	$(9.1 \pm .2) \times 10^{-6}$	"	$(1.2 \pm .2) \times 10^{-5}$	"
Promazine	150	$(1.5 \pm .2) \times 10^{-4}$	"	$(1.9 \pm .1) \times 10^{-6}$	"	$(2.9 \pm .2) \times 10^{-6}$	"
Secergan		$(1.2 \pm .1) \times 10^{-5}$	"	$(1.7 \pm .2) \times 10^{-5}$	"	$(1.5 \pm .2) \times 10^{-6}$	"

nitrogen, or the methyl group on the carbon α to $-\overset{+}{N}-(CH_3)_3$ group, modifies the interaction with the anionic site of ChE. Secergan was the strongest of the inhibitors for the AcChE-MeCh and the ChE-BuCh systems.

In Table 1 the compounds are listed in order of increasing recommended dosages. In general, inhibition of erythrocyte AcChE has little relationship to psychotropic activity. Inhibition of plasma ChE, on the other hand, appears to be inversely related to activity, if the recommended dosage is taken as an index to such activity. Thiopropazate, with the lowest suggested dosage, had the highest numerical value for K_i, indicating that, under the conditions used, it is a weaker inhibitor than the other compounds. The mechanism of inhibition by thiopropazate of plasma ChE, however, was found to be different from that of the other compounds tested (partially competitive rather than competitive), possibly because of the presence of the piperazine ring in the side chain of thiopropazate which could interact with a site different from the esteratic site.

Summary. Inhibition constants for 5 phenothiazines, chlorpromazine, promazine, secergan, thiopropazate and triflupromazine, using the systems human erythrocyte acetylcholinesterase with acetyl-β-methylcholine chloride as substrate and human plasma cholinesterase with both acetylcholine and butyrylcholine as substrates were determined. The mechanism of inhibition was of the mixed type for acetylcholinesterase, and of the competitive or partially competitive type for plasma cholinesterase. *In vitro* inhibition appeared to be unrelated to activity in the case of acetylcholine esterase and inversely related in the case of plasma cholinesterase.

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Effects of Psychotropic Compounds on Enzyme Systems, II. *In vitro* Inhibition of Monoamine Oxidase.* (26965)

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Our objective of attempting to correlate psychotropic action with effects on enzyme systems has been described(1). The significance of monoamine oxidase (MAO) and MAO inhibitors in relation to psychotropic compounds has been discussed by Zeller(2). We have adopted the method of Weissbach *et al.*(3) for determination of MAO, in order to obtain the necessary data for determination of K_i values(4).

Materials and methods. Kynuramine dihydrobromide was obtained from the Regis Chemical Co.; psychotropic compounds were supplied by the manufacturers, as listed in Table I. Livers of normal adult albino rats were homogenized in ice water and lyophilized. The lyophilized powder was stored in the refrigerator and served as the source of enzyme. No loss in activity was observed during storage for 3 months. The liver suspension was prepared fresh each day by homogenizing 250 mg of the powder in 6 ml of cold deionized water. This solution was kept at 0° throughout the day. Kynuramine dihydrobromide, 10 mg, was dissolved in 100 ml of H_2O. This solution was stored in the refrigerator. The compounds being tested were first prepared by dissolving 10 mg either in 25 ml of water or in 2 ml of ethanol and then adding water to the 25 ml mark. In the case of insoluble free bases, 0.1 ml of concentrated HCl was added to effect solution. After the preliminary run at an inhibitor concentration of 10 mg per 25 ml, the kinetic studies were made at such a concentration that significant differences were obtained among the tubes containing no inhibitors and those containing various amounts of inhibitor. To control pH, a 0.5 M phosphate buffer of pH 7.4 was used.

To each of twenty-two 18 × 150 mm Pyrex culture tubes, 0.1 ml of the liver suspension was added. A sufficient quantity of deionized water was added so that the final quantity of liquid in the tube was 6.0 ml. 0.3 ml of the phosphate buffer was placed into each tube. No inhibitor was added to the first 6 tubes, 0.5 ml to the next 5 tubes, 1.0 ml to the next 5 and 1.5 ml to the final 5 tubes. All tubes were thoroughly mixed at this point and the timer started. The first tube was used as a blank, therefore no kynuramine was added to it. To the first tube in each series (tubes #2, 7, 12 and 17), 0.5 ml of the kynuramine solution was added; 0.7 ml to the second tube; 1.0 ml to the third; 1.5 ml in the fourth and 2.0 ml in the last tube of each series. A tube containing all of the components except kynuramine, but including the highest level of inhibitor, was run to determine the contribution of the inhibitor to the observed optical density. After being thoroughly mixed, the tubes were incubated in a 37°C bath. They were read at 360 mμ in a Bausch and Lomb Spectronic 20 against the first tube as a blank after 5, 10, 20 and 30 minutes. All runs were made in duplicate.

After subtracting the contribution of inhibitor from each observed O. D., the O. D.s were adjusted, using a standard table(5). When these values were plotted, the velocity of the reaction (=V) was obtained from the slope. When Lineweaver-Burk plots(4) of 1/V vs. 1/S were made, the lines for each inhibitor concentration intercepted that for zero inhibitor concentration at the point for which $1/S = 0$. This would indicate competitive inhibition. To obtain K_i, the value for the quotient of the slope of the line at a given inhibitor concentration divided by the slope of the line with no inhibitor was plotted vs. the inhibitor concentration. The inhibitor concentration corresponding to a quotient of 2.00 was equal to K_i. For plotting the straight lines, a method of least squares was used.

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* Supported in part by grants from Nat. Inst. of Mental Health and New Mexico Heart Assn.

TABLE I. K_i Values for Inhibition of MAO by Selected Psychotropic and Related Compounds

Compound	Action	Mfr.	K_i (M)
Acepromazine	T	CB	No eff.
Adrenochrome	H	AH	1.8×10^{-4}
AF-365	T	AF	3.5×10^{-4}
Amitriptyline	E	M	2.8×10^{-5}
Anhalamine	H	P	No eff.
Anisoperidone	T	J	1.1×10^{-5}
Arecoline		P	2.0×10^{-3}
Benactyzine	T	M,MD	5.9×10^{-4}
Benztropine methane sulfonate		M	1.6×10^{-5}
BOL-148		S	2.7×10^{-4}
Butropipazone		J	3.7×10^{-5}
BW 58-271	T	BW	4.2×10^{-4}
CB-1497		CB	5.6×10^{-5}
CB-1519		CB	8.5×10^{-5}
CB-1613		CB	9.5×10^{-5}
CB-1619		CB	3.8×10^{-5}
CB-1620		CB	7.9×10^{-5}
CB-1658		CB	4.7×10^{-5}
Chlophedianol		R	5.6×10^{-5}
Chlordiazepoxide	T	HR	No eff.
Chlormethazanone	T	WI	No eff.
Chloroxazone		MC	2.7×10^{-6}
Chlorpromazine	T	SKF	9.9×10^{-3}
Chlorprothixen	T	HR	3.0×10^{-5}
Chlortrimeton		SC	2.4×10^{-4}
CI-383	T	PD	3.8×10^{-4}
CI-384	T	PD	2.7×10^{-4}
CI-400	H	PD	3.6×10^{-4}
Cqd-280		PH	4.7×10^{-4}
Cqd-285		PH	8.4×10^{-4}
Cqdd-280		PH	2.4×10^{-4}
Csat-131		PH	2.4×10^{-4}
Cyclohexalamine		PD	No eff.
Cyproheptadine		M	3.9×10^{-5}
Deanol	E	R	No eff.
Diethazine	T	RP	2.5×10^{-4}
Diethylpropion		MER	1.5×10^{-3}
Diphenylhydantoin	T	PD	No eff.
Diphenhydramine		PD	9.7×10^{-1}
Dyclonine		PM	2.3×10^{-6}
Ectylurea	T	A,U	No eff.
Ethychlorvynol	T	A	1.1×10^{-4}
Fluphenazine	T	SQ,WH	1.1×10^{-4}
Haloperidide	T	J	2.3×10^{-6}
Hexacyclonate		WL	No eff.
Hydroxyphenamate		AR	1.5×10^{-4}
Hydroxyzine	T	PF	2.8×10^{-5}
Imipramine	E	G	3.0×10^{-5}
IN-33	E	IN	No eff.
IN-399	T	IN	1.3×10^{-5}
Iproniazid	E	HR	1.2×10^{-5}
Isocarboxazide	E	HR	8.5×10^{-7}
Isopyrin		BG	No eff.
JB-318	H	LA	1.6×10^{-4}
JB-336	E	LA	4.6×10^{-4}
JB-835		LA	3.9×10^{-6}
KS-24		S	6.4×10^{-5}
KS-33		S	1.1×10^{-4}
KS-75		S	5.6×10^{-4}
Levomepromazine	T	LE	2.1×10^{-4}
Lidepran	E	RP	1.5×10^{-3}
Lysergic acid diethylamide	H	S	No eff.
Megimide	E	A	No eff.
Meperidide	T	J	3.9×10^{-6}
Mer 16		MER	2.2×10^{-5}
Methamphetamine	E	A,BW	9.8×10^{-5}
Methonalide	T	BR	No eff.
Methopromazine	T	LE	6.6×10^{-5}
Methylphenidate	E	CI	4.5×10^{-4}
α-methyltryptamine		U	2.1×10^{-4}
Nialamide	E	PF	5.8×10^{-6}
NP-207	T	S	8.4×10^{-5}
O.C. 1131-1417	T	M	5.2×10^{-5}
Olympax		D	1.4×10^{-4}
Orphenadrine	E	R	2.2×10^{-5}
Oxanamide	T	MER	No eff.
Pentylenetetrazol	E	K	1.2×10^{-3}
Petrichloral		IC	No eff.
Phenaglycodol	T	LI	4.4×10^{-4}
Phencyclidine	H	PD	1.5×10^{-4}
Phendimetrazine	E	AY	No eff.
Phenmetrazine	E	G	4.6×10^{-4}
Phenyltoxamine	E	BR	2.0×10^{-4}
Pipamazine	T	SE	No eff.
Piperazine pentanol		PD	2.9×10^{-4}
Piperidine phosphate		A	5.2×10^{-4}
Pipradrol	E	MER	5.8×10^{-4}
Prochlorperazine	T	SKF	1.2×10^{-4}
Profenamine	T	RP	5.8×10^{-4}
Promethazine	T	WY	1.5×10^{-4}
Propiomazine	T	WY	9.7×10^{-5}
Prothipendyl HCl	T	AY	6.5×10^{-5}
Psilocybin	H	S	No eff.
Pyrrolazote	T	U	8.1×10^{-5}
Rec 7-0268	E	REC	No eff.
Rescinnamine	T	PF	“
Reserpine	T	CI	Insol.
Riker 566	E	R	No eff.
Riker 594		R	“
RO 2-6797	E	HR	3.2×10^{-5}
RO 4-1027	E	HR	2.5×10^{-5}
RO 4-1038		HR	2.8×10^{-5}
RO 4-1340		HR	5.8×10^{-5}
RO 4-1385	E	HR	3.5×10^{-5}
RO 5-0994	E	HR	4.6×10^{-5}
RO 5-1162		HR	4.5×10^{-6}
RO 5-1221		HR	1.1×10^{-5}
S 9-888		PF	1.0×10^{-4}
SA-97		A	2.4×10^{-5}
SC-10600	E	SE	No eff.
Styramate		AR	3.4×10^{-4}
Tersavid		HR	3.1×10^{-5}
Tetrabenazine	T	HR	1.3×10^{-4}
Thiopropazate	T	SE	1.2×10^{-4}
Thioproperazine	T	RP	2.2×10^{-4}
Thymoxyalkylamine		DI	3.9×10^{-4}
Tricylamol		BW	2.5×10^{-5}
Trifluoperazine	T	SKF	6.9×10^{-5}
Trimeprazine	T	SKF	1.4×10^{-4}
Triperidol	T	J	4.8×10^{-5}
Win 2299		WI	4.0×10^{-4}
WV 357	T	DI	5.9×10^{-5}
WV 760	T	DI	3.7×10^{-4}
WV 770	T	DI	6.5×10^{-6}

ABBREVIATIONS:

E = energizer; H = hallucinogen; T = tranquilizer

A = Abbott Labs.; AF = Angelini Francesco; AH = A. Hoffer; AR = Armour Pharmaceutical Co.; AY = Ayerst Labs.; BR = Bristol Labs.; BW = Burroughs Wellcome and Co.; BG = Byk-Gulden; CB = Clin-Byla; CI = Ciba; D = Laboratoires Dausse; DI = Diwag Chemische Fabriken; G = Geigy Research Labs.; HR = Hoffmann-La Roche Inc.; IC = Ives-Cameron Co.; IN = Irwin, Neisler & Co.; J = Research Laboratorium Dr. C. Janssen; K = Knoll Pharmaceutical Co.; LA = Lakeside Labs.; LE = Lederle Labs.; LI = Eli Lilly & Co.; M = Merck Inst.; MC = McNeil Labs.; MD = Meco-Dumex; MER = Wm. S. Merrell Co.; P = S. B. Penick & Co.; PD = Parke, Davis & Co.; PF = Chas. Pfizer & Co.; PH = Philips-Duphar; PM = Pitman-Moore Co.; R = Riker Labs.; REC = Recordati Lab. Farmacol.; RP = Rhone-Poulenc; S = Sandoz Pharmaceuticals; SC = Schering Corp.; SE = G. D. Searle & Co.; SKF = Smith Kline & French Labs.; SQ = Squibb Inst. for Medical Research; U = Upjohn Co.; WH = White Laboratories; WI = Winthrop Labs.; WL = Warner-Lambert Research Inst.; WY = Wyeth Labs.

Results and discussion. Table I † lists K_i values obtained for a number of compounds.

Almost all of the compounds which were inhibitory exhibited competitive inhibition, at least at concentrations close to K_i. When some of these compounds were run at higher concentrations, the values could not be fitted to a straight line. However, this does not interfere with the calculation of K_i nor with the kinetic studies at the lower concentrations.

No obvious relationships between psychotropic action and MAO inhibition can be discerned from an examination of the data. Some of the compounds which were potent MAO inhibitors had tranquilizing properties while others acted as energizers; among the compounds with no MAO-inhibiting effects were tranquilizers, energizers and hallucinogens. Within a chemical series, however, there was some degree of correlation between potency and MAO K_i values. Thus, if one makes a table of phenothiazines arranged in order of K_i values and adds suggested dose levels, the compounds with lower K_i also have lower dose amounts (Table II).

TABLE II. MAO Inhibition and Dose Levels for Some Phenothiazines.

Phenothiazine	K_i (M)	Dose (mg)
Trifluoperazine	6.9×10^{-5}	4
Fluphenazine	1.1×10^{-4}	5
Prochlorperazine	1.2×10^{-4}	15
Thiopropazate	1.2×10^{-4}	15
Levomepromazine	2.2×10^{-4}	20-40
Diethazine	2.5×10^{-4}	20-50
Profenamine	5.8×10^{-4}	20-50
Chlorpromazine	9.9×10^{-3}	10-100
Acepromazine	No effect	150-200

Summary. K_i values for inhibition of MAO *in vitro* by 122 psychotropic compounds have been determined. At inhibitor concentrations close to or below the K_i value, the inhibition was competitive. MAO-inhibitors were found among tranquilizers, energizers and hallucinogens. The only correlation between MAO inhibition and psychotropic effects that could be found was within a chemical series.

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† A table listing structural formulae and other properties of these compounds and of other psychotropic compounds may be obtained from the authors.

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Leukemogenic Effect of Myleran on the Mouse Thymus. (26966)

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Of various alkylating agents with radiomimetic activity, Myleran (1,4-dimethanesulfonoxybutane) is particularly toxic to myelopietic cells(1). This property, which has led to the widespread use of Myleran in treatment of myeloid leukemia(2), suggested to us that the drug might be useful in conjunction with ionizing radiation for studying the pathogenesis of radiation-induced leukemia; *i.e.*, by means of Myleran, the marrow might be rendered aplastic before or after irradiation to determine the influence of myelopoietic activity on leukemia-induction. This premise formed the basis for the experiments herein reported.

Methods. Random-bred RF/Up male mice were exposed to 250 kvp whole-body X radiation at 10 weeks of age. The conditions of irradiation were identical to those reported earlier(3). Myleran was administered before or after irradiation (Table I), 20 mg/kg intravenously in a solution of dimethylacetamide, prepared according to the method of Weston *et al.*(4, and personal communication). The timing of Myleran administration in relation to irradiation was adjusted primarily so that the marrow was irradiated either at time of maximum aplasia (5 days after Myleran) or maximum regenerative hyperplasia (15 days after Myleran), as judged from preliminary histologic studies of mice treated identically. For comparison, irradiation was also carried out 5 days before Myleran administration. Control groups were sham-irradiated and injected with dimethylacetamide alone (the Myleran vehicle). After treatment, the animals were caged in groups of 9-10, with free access to Purina laboratory chow and drinking water, and observed until natural death. A necropsy was performed on each animal, with histologic examination as needed to confirm gross diagnosis.

Results. Mortality within the first 30 days after treatment was negligible in all groups. Exposure to X-rays shortened the ultimate life span, however, and increased the incidence of myeloid leukemia and thymic lymphosarcoma, the effects of combined treatment with X-rays and dimethylacetamide (Table I) closely approximating those obtained previously with X-rays alone(3). Myleran, whether administered alone or in conjunction with X-rays, reduced survival time and incidence of myeloid leukemia and increased the incidence of thymic lymphoma, but the magnitude of these effects varied with conditions of treatment (Table I); *i.e.*, it was most pronounced when Myleran was administered 15 days before irradiation (Fig. 1). The Myleran vehicle, dimethylacetamide, was without significant effects on the life span or incidence of these neoplasms. Incidence of leukemias other than the myeloid and thymic types tended to be lower among irradiated mice than among controls, in agreement with earlier observations(3), and was not significantly affected by Myleran. The mean age at death with all observed diseases was reduced by irradiation, in keeping with previous findings(3).

Discussion. The co-leukemogenic, and possibly leukemogenic, effects of Myleran on the thymus were not anticipated at the outset of the study. Instead, it was expected that Myleran would probably enhance induction of myeloid leukemia because of its toxicity to the marrow, an effect opposite to that obtained. This paradox is yet to be explained, but several factors deserve consideration.

First, it is conceivable that Myleran caused too much injury to myelopoietic cells to permit myeloid leukemogenesis, since excessive injury by X-rays alone (*i.e.*, doses above 400 r) inhibits the induction of the disease(3). The preponderantly higher incidence of myeloid leukemia in the Myleran-treated mice at 300 r than at 150 r, however, is inconsistent with the idea that the inhibition resulted from toxicity alone. Furthermore, the inhibitory effect of Myleran varied with time of administration, being maximal when the drug was given 15 days before irradiation. Another possible explanation for the inhibition was the high frequency of Myleran-induced thymic lymphosarcoma, since the incidence of this disease tended to be inversely related to the incidence of myeloid leukemia. Statistical adjustment of the myeloid leukemia incidence to correct for this possibility, however, failed to restore it to expected levels (Table I). An additional explanation of the effects observed when Myleran was given 15 days before irradiation is that Myleran may have increased the number of myeloblasts surviving irradiation by increasing the total number irradiated; *i.e.*, the marrow was rendered maximally hyperplastic at time of irradiation. This mechanism fails, however, to account for the effects of Myleran when given after irradiation.

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* Operated by Union Carbide Corp. for the U. S. Atomic Energy Commission.

Equally puzzling is the co-leukemogenic action of Myleran on the thymus, which also varied in relation to the time between Myleran administration and irradiation. Owing to the protective activity of intact marrow in inhibiting radiation-induced thymic tumorigenesis(see 6), and the co-leukemogenic action of urethane when given in conjunction with X-rays and marrow shielding(7), the effects of Myleran may be postulated to have resulted from marrow toxicity. It is noteworthy, however, that the action of Myleran was maximal when the drug was given 15 days before irradiation, whereas in the case of urethane, co-leukemogenic activity is minimal when the agent is given before irradiation(8). Although the 2 agents thus far have not been compared under identical experimental conditions, the data suggest that the leukemogenic effects of urethane and Myleran differ in mechanism. Thus, a more detailed analysis of the leukemogenic activity of the 2 agents in relation to the pharmacologic and toxicologic effects is called for.

We made an effort to ascertain whether Myleran alone caused thymic involution and whether it would impair regeneration of the thymus when given in conjunction with X-rays under the conditions of this experiment. This inquiry was prompted by observations

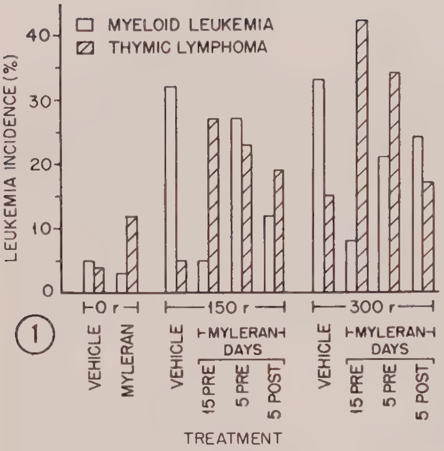
TABLE I. Leukemia Incidence as Affected by Myleran and X-Rays.

			Mice dying with myeloid leukemia			Mice dying with thymic lymphoma			Mice dying with other leukemia	
			Incidence			Incidence				
Treatment	No. of mice	Mean age at death (mos)	Observed (%)	Adjusted[a] (%)	Mean age at death (mos)	Observed (%)	Adjusted[b] (%)	Mean age at death (mos)	Incidence observed (%)	Mean age at death (mos)
None (control)[c]	314	19.1	3	4 (± 1)	16.3	5	5 (± 3)	15.5	29	20.6
Vehicle	71	20.1	5	5 (± 4)	16.0	4	4 (± 4)	18.0	45	19.2
Myleran	59	17.2	2	2 (± 2)	20.0	12	12 (± 5)	10.9	39	19.0
150 r + vehicle	74	16.0	32	33 (± 5)	15.5	5	6 (± 4)	10.5	23	16.4
150 r + Myleran 15 days before	67	14.9	5	8 (± 5)	13.3	27	28 (± 6)	9.0	29	17.1
" " " 5 " "	71	13.6	27	34 (± 6)	13.4	23	26 (± 6)	10.1	19	15.2
" " " 5 " after	69	14.8	11	14 (± 6)	15.4	19	20 (± 6)	9.7	32	15.6
300 r + vehicle	70	13.7	33	38 (± 5)	12.8	15	17 (± 6)	9.4	18	17.7
" " Myleran 15 days before	70	12.6	8	14 (± 7)	12.3	42	44 (± 7)	9.5	20	16.1
" " " 5 " "	71	13.6	21	31 (± 7)	13.0	34	38 (± 4)	9.4	14	14.7
" " " 5 " after	74	15.2	24	28 (± 6)	13.7	17	19 (± 6)	11.5	23	16.6

[a] Observed incidence adjusted(5) to correct for loss of animals attributed to mortality from thymic lymphoma (standard error in parentheses).

[b] " " " " " " " " " " " " Myeloid leukemia " " " "

[c] Controls from earlier experiments(3).



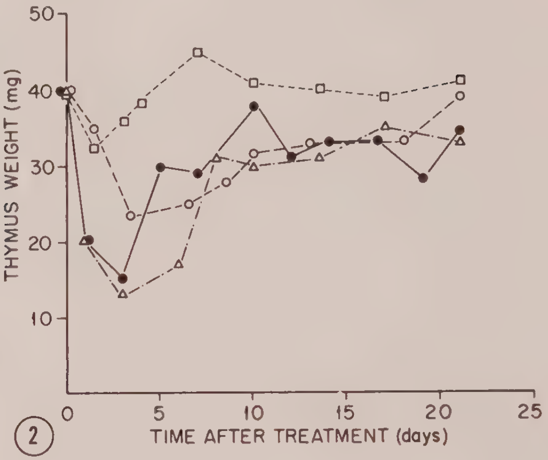


FIG. 1. Observed incidence of myeloid leukemia and thymic lymphoma as influenced by time of Myleran injection in relation to irradiation.

FIG. 2. Influence of Myleran on thymus weight and restoration of thymus weight after irradiation (each point represents data for 6 mice; see text for details of treatment).
□ vehicle
○ Myleran, 20 mg/kg
△ 300 r whole-body X-irradiation
● Myleran, 20 mg/kg, 15 days before 300 r whole-body X-irradiation

that thymic tumorigenesis appears to be inversely correlated with the rapidity of restoration of thymus weight after irradiation(9) and that urethane decreases the ability of the marrow to promote regeneration of the irradiated thymus(10). From the limited data available (Fig. 2), there is no evidence that Myleran adversely affected thymus regeneration at the dose level used in this study, although it did cause transient atrophy. Since, however, larger doses of Myleran have been observed to cause more profound and long-lived thymic atrophy(11), the limited thymic weight data presented herein should not be regarded as conclusive.

Summary. Myleran given before or after whole-body X-irradiation increased the incidence of thymic lymphomas and decreased the incidence of myeloid leukemias in male mice of the RF strain. The magnitude of these effects varied, depending on the time Myleran was administered in relation to irradiation. Effects were greatest when Myleran was given 15 days before exposure to X rays.

These results are paradoxical in view of the myelotoxic action of Myleran and attest further to the complexity of factors influencing susceptibility to radiogenic leukemia.

We are grateful to A. W. Kimball and M. A. Kastenbaum for statistical analysis of the data, to W. D. Gude for histological assistance, and to Burroughs Wellcome Co., Inc., for supplying Myleran used in this study.

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Behavior of Different Geographic Strains of *Schistosoma japonicum* as Determined by the Circumoval Test.* (26967)

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Species specificity of *Schistosoma haematobium, S. mansoni,* and *S. japonicum* has been demonstrated serologically by the circumoval test(1). As *S. japonicum* consists of at least 4 distinct geographic strains, Chinese, Formosan, Japanese, and Philippine(2), it is of interest to know whether these 4 strains are distinct in the circumoval test.

Materials and methods. Four geographic strains of *S. japonicum* were used: the Chinese (C), Formosan (F), Japanese (J), and Philippine (P). The cercariae of the C strain were obtained from *Oncomelania hupensis* originating in Kashing, Chekiang, China; the F strain, from *O. formosana* obtained from Changhua, Taiwan, China; the J strain, from *O. nosophora* obtained from Kofu, Japan; and the P strain, from *O. quadrasi* obtained from Leyte, in the Philippines.

Eight rabbits divided into 4 groups of 2 each were infected by the cutaneous route with the 4 strains of *S. japonicum*. Each rabbit was infected with 70 male and 70 female cercariae of a strain of the parasite and was bled on the 70th day after schistosome eggs were found in the stools. The sera obtained from these 8 rabbits were the antisera (AS) used in the circumoval test. Sera from 2 normal rabbits were used as controls. The antisera and normal sera (NS) were inactivated at 56°C for 30 minutes before use.

The eggs of *S. japonicum* were obtained from the liver of infected mice. Twenty-four mice divided into 4 groups of 6 each were infected with the 4 strains of *S. japonicum*. Each mouse was exposed to 100 cercariae of balanced sexes of one strain of *S. japonicum*. These mice were sacrificed on the fifth day after eggs were found in stools. The livers of the mice were ground and schistosome eggs were then concentrated by repeated natural sedimentation with normal saline. The last concentration was accomplished by centrifugation.

Single drops containing these centrifuged eggs were then mixed with single drops of either antiserum or normal serum on a slide. These serum-egg mixtures were then covered with 22 mm square coverglasses and rimmed with paraffin. The slides were kept at 37°C in an incubator, and the results were read after 24 hours' incubation. Only the eggs (E) which contained active mature miracidium were recorded as the number of eggs examined in this test. The antigen-antiserum precipitates which appeared on the egg surface were carefully differentiated from the other precipitates noted occurring with normal serum. Only the former were recorded as eggs with positive reactions. As the degrees of the positive reactions are prone to subjective errors, they were not classified in this study.

In the accompanying table, the following abbreviations are used: CE refers to eggs of Chinese strain; FE, the Formosan strain; JE, the Japanese strain; PE, the Philippine strain. CAS refers to antiserum of the Chinese strain; FAS, the Formosan strain; JAS, the Japanese strain; and PAS, the Philippine strain.

Results. Twenty sets of egg-serum tests, divided into 4 series, were made. The results

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TABLE I. Percentage of eggs of 4 geographic strains of *S. japonicum* showing positive circumoval precipitation in homologous, heterologous, and normal sera.

Series	Eggs vs serum	No. of eggs	Eggs with positive reactions No.	%
CE	CE vs CAS	215	129	60.0
	CE vs FAS	175	59	33.7
	CE vs JAS	282	127	45.0
	CE vs PAS	168	42	25.0
	CE vs NS	100	0	.0
FE	FE vs CAS	777	248	31.9
	FE vs FAS	606	461	76.0
	FE vs JAS	781	263	33.7
	FE vs PAS	447	57	12.8
	FE vs NS	100	0	.0
JE	JE vs CAS	274	109	39.8
	JE vs FAS	288	93	32.3
	JE vs JAS	310	235	75.8
	JE vs PAS	177	68	38.4
	JE vs NS	100	0	.0
PE	PE vs CAS	109	39	35.8
	PE vs FAS	100	32	32.0
	PE vs JAS	150	85	56.7
	PE vs PAS	120	82	68.3
	PE vs NS	100	0	.0

were shown in Table 1. In all of the 4 series, eggs with normal serum showed no reaction. The percentages of eggs showing positive reactions were much higher with the homologous antiserum than with the heterologous antisera. The difference between the percentage of the eggs showing positive reactions with the homologous antiserum and the percentages with the heterologous antisera was found to be significant at the 5 % level in each series by the chi square test.

Discussion and conclusion. Although the circumoval reaction is species specific for *S. japonicum*, the results of the present studies show that with this test, there were cross reactions among the 4 geographic strains. A significantly larger proportion of eggs showing positive circumoval precipitation, however, was seen when the eggs were incubated with the antisera of the heterologous strains. This indicates that the geographic strains of *S. japonicum* have already become partly distinct in their serological characteristics.

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Isomerized Fat and Serum Cholesterol in Swine.* (26968)

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Although the effect of saturation of fatty acids on serum cholesterol levels in a variety of experimental animals and in man has been studied extensively, no investigation dealing with fatty acid isomerization in this relationship has been noted. The present study was designed to measure the relative effects of natural (cis) fats and hydrogenated fat which contains isofatty acids including transisomers on serum cholesterol level. In addition relatively pure triolein (cis) was compared with trielaidin (trans).

Experimental. Adult sows, ranging in age from approximately 3 to 7 years and in weight from 300 to 500 lbs, were used as the experimental animal. A purified diet (composition shown in Table I) was fed at a constant level for each animal in an amount that just maintained body weight. The diet was mixed with water to form a slurry and was fed in 2 equal portions during each day. Extra water was provided only at feeding times. The pigs were housed individually in a closed barn, but temperature was not constant. Body weight was measured weekly.

The pattern of feeding was to give a low fat diet for 3 weeks followed by 3 weeks on a diet containing the test fat at a level of

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TABLE I. Diet Composition.

	Basal low fat %	Experimental test fat %
Soy protein (Promine)	14.9	18.8
Glucose (Cerelose)	76.8	49.2
Corn oil (Mazola)	2.0	2.5
Test fat	—	21.5
Cellulose (Solka-Floc)	2.0	2.5
Minerals*	4.0	5.0
Fat sol. vitamins†	+	+
Water sol. vitamins‡	+	+
Choline	.3	.3
	100.0	99.8
Total Calories (calculated)	395	500
Ratio of amount of diet fed	1.27 g	1.00 g

* Minerals in g/100 g diet: $CaHPO_4 \cdot 2H_2O$, 0.77; $CaCO_3$, 1.05; KH_2PO_4, 1.15; NaCl, 0.50; $CuSO_4 \cdot 5H_2O$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.165; KI, trace, $MnSO_4 \cdot H_2O$, 0.123; $CoCl_2 \cdot 6H_2O$, 0.00008; $MgCO_3$, 0.38; $ZnSO_4 \cdot 7H_2O$, 0.48.

† Fat sol. vit. mg/100 g diet dissolved in 2 g corn oil: vit. A acetate, 4.472; calciferol, 0.0135; α tocopherol, 18.0

‡ Water sol. vit. mg/100 g diet: thiamine HCl, 0.14; riboflavin, 0.27; niacin, 1.12; Ca pantothenate, 1.10; pyridoxine, 0.16; vit. B_{12}, 0.013; folic acid, 0.067; menadione, 0.112.

40% of total calories. Extra carbohydrate was supplied during the low fat period so that total calorie intake was constant. The groups of pigs were thus fed in 3-week periods so that a low fat period preceded and followed a given test fat period. Blood was obtained at weekly intervals by clipping of the end of the tail. Serum total cholesterol was determined on duplicate 0.3 ml samples by the method of Abell *et al.*(1). Cholesterol values for the second and third week of each period were averaged for the results given in Table II. An attempt was made to use the same pigs for all of test fats. However, the experiment ran for many months and some pigs had to be discarded because of degenerative disease or lack of appetite. When new pigs were introduced they were kept on the low fat diet for approximately 2 months and were then introduced into the study only if serum cholesterol values were reasonably constant and fell into the range exhibited by the experimental animals. The number of pigs used varied from 10 to 7 as shown in Table II.

The hydrogenated fats were specially prepared from soybean oil so as to have iodine numbers in the range of 105 or 80. Two separate lots of each hydrogenated fat (described as hyd. fat No. 1 or No. 2) were supplied through the courtesy of Dr. Fred Mattson, Proctor and Gamble Co.

The various lots of fatty acids were methylated by the technique described by Roper and Ma(2). The different batches of hydrogenated fat were analyzed for fatty acid composition by gas chromatography using a Barber and Coleman Model 10 gas chromatographic instrument. A 6 ft. column packed with diethylene glycol succinate (DEGS) in a ratio of 1:5 w/w on Celite 545, 80-100 mesh, was maintained at 180°C with Argon used as the Carrier gas at a flow rate of 59

TABLE II. Serum Cholesterol Values in Swine Fed Experimental Fat Diets.

Fat component in diet	No. animals	Serum Cholesterol Low fat control periods	 Fat periods	 Increase
		mg/100 ml	mg/100 ml	mg/100 ml
#1 Hyd. Fat I.V. 105–110	10	67.2 ± 1.4*	86.6 ± 2.1	19.4 ± 2.1
Fat Mixture A	10	67.0 ± 1.5	85.6 ± 2.5	18.6 ± 2.5
#1 Hyd. Fat I.V. 70-80	10	68.8 ± 1.6	93.4 ± 2.6	24.2 ± 2.6
Fat Mixture B	10	69.8 ± 1.0	90.4 ± 1.7	20.6 ± 1.7
#2 Hyd. Fat I.V.-105	8	66.2 ± 3.2	88.8 ± 2.2	22.6 ± 2.2
Fat Mixture C	7	68.9 ± 3.3	85.3 ± 2.1	16.4 ± 2.1
#2 Hyd. Fat I.V.-80	8	67.2 ± 3.1	91.0 ± 3.5	23.8 ± 3.5
Fat Mixture D	7	67.3 ± 3.0	98.0 ± 3.6	30.7 ± 3.6
Triolein	8	67.0 ± 1.2	93.9 ± 3.1	26.9 ± 3.1[+]
Trielaidin	8	69.4 ± 1.2	87.4 ± 2.0	18.0 ± 2.0

* Mean ± standard error.

[+] Significant at 0.05 probability.

TABLE III. Saturated and Unsaturated Fatty Acid Content of Dietary Fats.

Fats	Sat. fatty acid (palmitic & stearic)	Monoene (palmitoleic & oleic)	Diene (linoleic)	Triene (linolenic)
#1 Hydrogenated Fat (I.V. 108-110)	15.1	48.8	35.5	.6
Fat mixture A	14.6	55.3	29.7	—
" " C	13.1	50.2	36.7	—
#1 Hydrogenated Fat (I.V. 70-80)	22.2	63.0	14.8	—
Fat mixture B	18.1	62.4	19.6	—
" " D	15.2	72.7	11.7	.3
#2 Hydrogenated Fat (I.V. 105)	17.9	48.0	32.8	1.2
Fat mixture CN	11.2	50.7	37.7	.4
#2 Hydrogenated Fat (I.V. 80)	21.9	67.0	11.1	—
Fat mixture DN	21.6	68.3	7.3	—
				Ratio cis/trans
Triolein	3.3	87.7	2.7	2.84/ .70
Trielaidin	3.1	89.2	2.0	.70/2.76

ml per min. An applied potential of 500 volts was maintained on the ionization detector cell containing 56 μc of Radium-226. The cell bath was heated to 240°C, while the flash heater was maintained at 287°C. This technique was found to have a standard error of estimate of ± 0.25%.

The combined values for palmitic and stearic and for palmitoleic and oleic together with values for dienes and trienes are given in Table III. Natural oils and fats were similarly analyzed and mixtures made so as to simulate the fatty acid composition of the hydrogenated fats. Fat mixtures A and B were made from cottonseed oil (Wesson oil) and olive oil. These were intended to correspond to batch No. 1 hydrogenated fat, iodine No. 105-110 and 70-80 respectively. Fat mixture C was composed of safflower oil and olive oil and D was safflower oil and beef tallow. These were intended to correspond to batch No. 2 hydrogenated fat, iodine No. 105 and 80 respectively. The fatty acid analyses of these mixtures are shown in Table III.

A purified commercial "triolein" was elaidinized and re-esterified with glycerol to give a "trielaidin" (prepared by the Aero Chemical Corp., Newark, N. J.). Fatty acid analyses for these 2 preparations are given in Table III. The 2 monoenes were analyzed for relative cis- and trans-isomerization by infrared spectroscopy(3). The "triolein" and the "trielaidin" gave a ratio of cis to trans of 2.84:0.70 moles per kg and 0.70:2.76 moles per kg respectively.

Results and discussion. There were no statistically significant differences between any pair of fats except the triolein and trielaidin. The natural form (triolein) gave slightly higher cholesterol values than the unnatural form (trielaidin). The more saturated hydrogenated fat of batch 2 had a natural fat mixture for comparison that was a closer approximation of fatty acid composition than was the case with batch 1. In this pair the natural mixture also gave a slightly higher but not significantly different serum cholesterol. The one clear-cut effect that was noted was that triolein had a definite serum cholesterol increasing effect. This might have been surmised from the olive oil feeding experiments in swine reported by Barnes *et al.*(4), but differs from results obtained in man(4,5). In this respect cholesterol responses of the pig more closely resemble observations made in the rat(6).

Trans-fatty acids are absorbed and deposited in the tissues; furthermore, they appear to be metabolized(7). Perhaps the most striking difference is the complete lack of activity of trans-linoleic acid as an essential fatty acid and even the possibility of certain trans acids being essential fatty acid antagonists(8). The results obtained in the present study do not rule out the possibility of a small difference in the effect of cis- and trans-isomers on serum cholesterol. However, they do not indicate any major influence of isomerization such as would be obtained in commercial hydrogenation on the serum cholesterol level.

Summary. Adult sows were fed a purified diet and in alternate 3-week periods were fed the diet either with a low fat or a high fat (40% of calories) content. In the high fat periods hydrogenated fats were compared with natural plant fat mixtures designed to have the same fatty acid composition. The difference in the fats studied was in their content of isomerized fatty acids. Also triolein was compared with trielaidin. The hydrogenated fats gave the same serum cholesterol response as their corresponding natural plant fat mixtures. Trielaidin gave a lower ($P < 0.05$) serum cholesterol response than triolein.

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Effect of "Sex" of Tissue Cultures on Growth of Encephalomyocarditis Virus.* (26969)

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The fact that the sex of the host may condition both susceptibility and response to viral infection has been established for naturally-occurring poliomyelitis(1,2). That this is also true in experimentally-produced disease has been noted by Weinstein and Chang (3) who found that the LD_{50} of several viruses was influenced, to a varying degree, by the sex of the animal. Male mice were observed to be much more susceptible to encephalomyocarditis virus, females were less resistant to poliovirus, while little or no sex differences were demonstrable with the agents of influenza and vaccinia.

These observations, together with the demonstration that cells derived from males and females may multiply at different rates(4,5), suggested the possibility that degree of susceptibility of isolated tissues to viral invasion might be influenced by the sex of the animal from which they were derived. This paper reports the results of studies with encephalomyocarditis virus which indicate that the "sex" of the mouse muscle culture into which it is inoculated, determines rate and extent of its growth.

Materials and Methods. Cultures of muscle of mature male and female mice were prepared by a previously described method(6) and grown in a medium consisting of 10% horse serum, 5% beef embryo extract, 45% bovine amniotic fluid, and 40% Hanks' solution.

A 10% mouse brain pool of encephalomyocarditis virus was titrated for LD_{50} by intraperitoneal injection into 6-week old animals, and stored at —20°C until used. The muscle cultures were inoculated with varying doses of the brain-virus preparation. Only those tubes showing good growth of cells and

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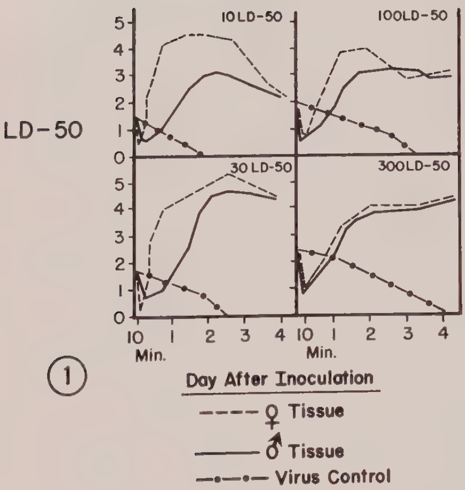

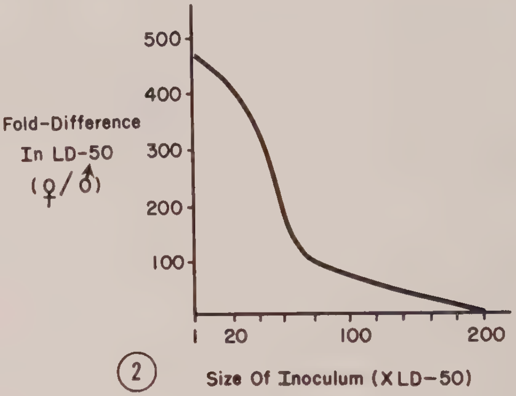

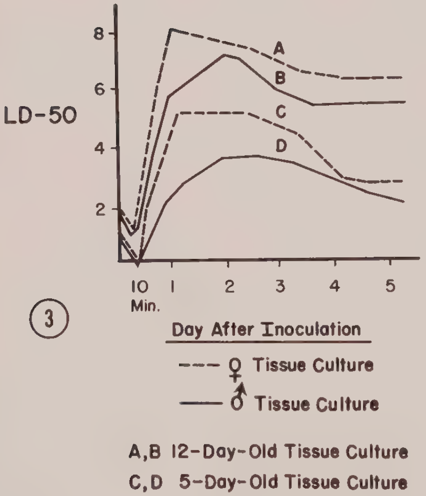

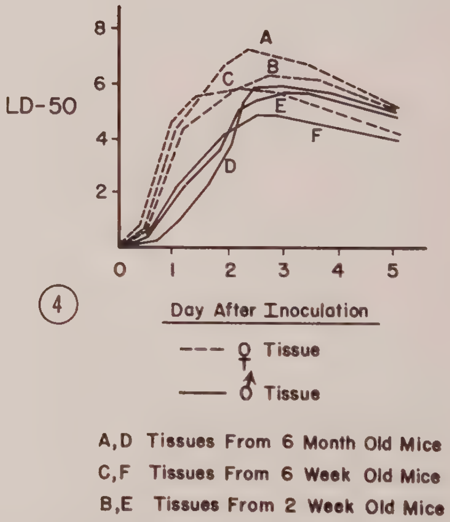

FIG. 1. Relation of size of inoculum to growth of EMC virus in male and female tissues.

FIG. 2. Ratios of degree of growth of EMC virus in male and female tissues.

FIG. 3. Effect of age of tissue culture on growth of EMC virus in male and female cells.

FIG. 4. Effect of age of mice from which muscle was obtained on growth of EMC virus.

in which the degree of multiplication of the muscle from both sexes was approximately the same were infected; 10 tubes of "female" and "male" tissue were used for each experiment which was repeated at least 3 to 4 times. The cultures were incubated in a stationary position at 37°C. Aliquots (0.1 ml) were removed from each tube after 24, 48, 72, 96, 120, and 144 hours and stored at —20°C until titrated.

Virus growth curves were determined by titration in animals. The fluid removed after varying periods of incubation was diluted by 10-fold steps and 0.2 ml of each dilution injected intraperitoneally into 4 to 6 week old male mice; 6 animals were used for each dilution. The LD_{50} was calculated each day until the end of experiment; deaths occurring in the first 24 hours were not included.

Results. As can be seen in Fig. 1, the rate and extent of growth of encephalomyocarditis virus were different in cultures of "male" and "female" muscle. Degree of difference was dependent on size of the inoculum with which the tissues were infected. It was maximal when the quantity of virus used was small and decreased to the vanishing point as this was increased. Thus, with an inoculum of 10 LD_{50}, rate of multiplication and total virus yield during the first 2 days of incubation were greater in "female" cultures while with an infecting dose of 300 LD_{50} no difference between the sexes was noted. These observations were confirmed in another experiment in which inocula varying from 1 to 200 LD_{50} were employed. Data calculated on the basis of the female/male difference are presented in Fig. 2 in which it can be seen that the yield of virus was almost 500 times higher in "female" tissue when the inoculum was 1 LD_{50} and that, with 200 LD_{50}, no difference was present.

Since uninfected cultures of cells from male and female tissues may grow at different rates (4,5), a study was carried out to determine whether this was responsible for the observations described above. Cultures of muscle from male and female mice were grown for 5 and 12 days respectively; degree of cellular multiplication was greatest in the latter. Tubes from each period of growth were selected so that the number of cells in the "male" and "female" ones were approximately the same and all were infected with 10 LD_{50} of encephalomyocarditis virus. Aliquots were removed every 24 hours during 5 days of incubation at 37°C and titrated by intraperitoneal injection into mature male mice. The virus was found to grow at a more rapid rate and to a greater extent in "female" cells of both the 5- and 12-day old tissues (Fig. 3). Despite the fact that the speed and degree of viral replication were less in the younger cells, the differences between female and male cultures in relation to multiplication of virus were greater in them than in the older ones.

The data presented in Fig. 4 indicate that age of the animal from which the muscle used for infection was derived played some part in determining the magnitude of viral multiplication. Mice of both sexes, aged 6 months, 6 weeks, and 14 days respectively were used in these studies. The muscles of the male and females in each of these groups were cultured. Tubes exhibiting approximately the same degree of cell growth were selected, inoculated with 10 LD_{50} of EMC virus, and incubated at 37°C. Aliquots were withdrawn once daily for 5 days and LD_{50} determined by inoculating varying dilutions intraperitoneally into 6-week old male mice. Although the virus was found to multiply more rapidly and to a greater degree in "female" muscles of all age groups, the differences from the males were greatest in the 6-month old animals and least in the 2-week old ones. When titrated after 24 hours of incubation, the quantity of virus present in cultures of muscle of 6-month old mice was found to be 5700 times greater than could be demonstrated in "male" muscle of the same age; the female to male ratio of virus in tissue of 2-week old animals was only 8 to 1.

Discussion. These results indicate that susceptibility to viral invasion is conditioned by the sex of the host at all levels of complexity of cellular organization ranging from the whole animal to isolated tissue growing in artificial media. The fact that differences in growth of encephalomyocarditis virus in "female" and "male" cells were greater in muscle removed from fully mature animals raises the possibility that the determinant factor was the influence of sex hormones during life. However, a similar effect of lesser degree was also noted in cultures of the tissues of sexually-immature mice. This suggests that the basic mechanism involved in determining sex differences in susceptibility of cells to virus infection is not endocrine activity alone, although this may influence quantitative aspects of the phenomenon.

The possibility that variations in extent of cell growth present at the time of viral infection may have been responsible for the observed differences in susceptibility to virus invasion of male and female tissues merits consideration. The data, however, do not support this hypothesis for the following reasons: (a) At the time of infection with EMC virus, the number of cells in the cultures of both the male and female muscle were approximately equal. (b) Despite the fact that there were fewer cells in 5-day than in 12-

day old cultures of mouse muscle, the differences in speed and extent of growth of virus were greatest in the younger tissues. If degree of viral multiplication were dependent only on the number of muscle cells available for invasion, the female to male ratio of virus should have been highest in the 12-day old cultures because they contained a greater number of cells than the 5-day old ones at time of infection.

It is striking that differences in degree of growth of EMC virus in cultures of muscle from male and female mice could be demonstrated only with inocula of certain size. With small dose of virus, the difference between the 2 sexes was maximal; as quantity of infecting agent was increased, it became less marked until it was no longer noted when 200 to 300 LD_{50} were used. The reason for this phenomenon is not apparent.

Summary. When EMC virus was grown in cultures of mouse muscle, it multiplied more rapidly and to a greater degree in the tissue obtained from female animals. The quantity of virus used to produce infection was an important determinant in demonstration of this phenomenon. The sex differences did not appear to be related to the number of cells present in the muscle culture at time of viral inoculation.

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Hemagglutination-Inhibition Antibody Responses in Human Adenovirus Infections. (26970)

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Although neutralizing antibody responses in persons infected with adenoviruses tend to be type-specific, heterotypic responses occur frequently(1). Furthermore, because of certain unusual aspects of adenovirus neutralization tests it is difficult to equate the findings from different laboratories or even those obtained in the same laboratory at different times(1). Complement-fixing (CF) antibody responses generally show no type-specificity in human adenovirus infections(1).

Preliminary data indicating that adenovirus hemagglutination-inhibition (HI) antibody responses in man might be type-specific have been presented(2). This report is concerned with additional data subsequently obtained from a study of paired sera from 36 children naturally infected with adenovirus types 1, 2, 3, or 5 and from 27 adults naturally infected with adenovirus types 4 or 7.

Materials and methods. The children with adenovirus type 1, 2, 3, or 5 infections were residents of an institution (Junior Village) and were part of a population whose microbial experiences were the subject of intensive study(3). Paired sera were selected so that they spanned the first known isolate of a given adenovirus serotype in a child, yet did not include adenoviruses of other serotypes. The first serum of each pair antedated the adenovirus isolate and second was collected at varying times thereafter as indicated in the tables.

The adults with adenovirus type 4 or 7 infections were naval recruits from whom these particular serotypes were isolated during a study of respiratory illness. The first serum from these individuals was obtained at onset of illness; the second was collected approximately 21 days later.

HI tests were carried out exactly as has been described previously(4) using prototype strains of the various serotypes. In brief, rhesus erythrocytes were used for adenovirus types 3 and 7 and rat erythrocytes for types 1, 2, 4, and 5. All sera were treated with kaolin and adsorbed with rhesus and rat erythrocytes. Erythrocytes were al-

TABLE I. Hemagglutination-inhibition and Complement-fixing Antibody Responses of 8 Children Infected with Adenovirus Type 1

Age of child in mo. at time of virus isolation	Days after virus isolation 2nd serum collected	HI titer with indicated antigen						
		T1	T2	T3	T4	T5	T7	CF titer
9	27	<10*	<10	<10	<10	<10	<10	<8
		40	<10	<10	<10	<10	<10	32
12	36	<10	<10	40	<10	<10	<10	16
		10	<10	20	<10	<10	<10	64
12	36	80	<10	<10	<10	20	<10	8
		80	<10	<10	<10	<10	<10	64
12	78	<10	<10	80	<10	<10	<10	32
		80	<10	160	<10	<10	<10	64
22	44	10	80	20	<10	10	<10	64
		40	80	20	<10	10	<10	128
23	51	<10	<10	<10	<10	20	<10	8
		80	<10	10	<10	80	<10	64
33	35	<10	<10	<10	<10	<10	<10	16
		80	<10	<10	<10	<10	<10	32
37	30	<10	40	<10	<10	<10	<10	32
		20	40	<10	<10	<10	<10	128

* In this and subsequent tables the first row of data pertains to first serum specimen, the second row to second serum specimen from a given individual.

TABLE II. Hemagglutination-inhibition and Complement-fixing Antibody Responses of 9 Children Infected with Adenovirus Type 2

Age of child in mo. at time of virus isolation	Days after virus isolation 2nd serum collected	HI titer with indicated antigen						
		T1	T2	T3	T4	T5	T7	CF titer
8	36	<10	10	<10	<10	<10	<10	<8
		<10	20	<10	<10	<10	<10	<16
11	83	10	<10	20	<10	<10	<10	32
		10	80	<10	<10	<10	<10	64
12	30	80	10	<10	<10	≧320	<10	<8
		80	≧320	<10	<10	≧320	<10	16
16	117	20	160	10	<10	40	<10	16
		80	≧320	<10	<10	20	<10	16
18	43	20	160	20	<10	<10	20	32
		20	160	<10	<10	<10	10	32
22	57	40	<10	20	<10	<10	<10	16
		40	20	10	<10	<10	<10	32
27	44	<10	≧320	<10	<10	<10	<10	<8
		<10	160	<10	<10	<10	<10	<16
28	76	<10	80	40	<10	40	<10	NT*
		40	≧320	<10	<10	80	<10	NT
38	62	160	<10	<10	<10	80	<10	16
		160	80	<10	<10	80	<10	32

* In this and subsequent tables NT indicates that sera were not tested for CF antibodies because one or both specimens were anticomplementary.

TABLE III. Hemagglutination-inhibition and Complement-fixing Antibody Responses of 9 Children Infected with Adenovirus Type 3

Age of child in mo. at time of virus isolation	Days after virus isolation 2nd serum collected	HI titer with indicated antigen T1	T2	T3	T4	T5	T7	CF titer
8	43	20	<10	<10	<10	<10	<10	NT
		40	<10	20	<10	<10	<10	NT
10	22	<10	<10	<10	<10	<10	<10	<8
		<10	<10	10	<10	<10	<10	32
11	48	40	<10	<10	<10	<10	<10	16
		20	20	20	<10	<10	<10	32
15	28	≥320	<10	<10	<10	<10	<10	64
		≥320	<10	20	<10	<10	<10	64
18	26	<10	80	<10	<10	<10	<10	16
		<10	80	20	<10	<10	<10	64
18	29	<10	<10	<10	<10	<10	<10	<8
		<10	<10	40	<10	<10	<10	<16
18	29	160	<10	<10	<10	160	<10	32
		≥320	<10	20	<10	≥320	<10	128
31	43	<10	<10	<10	<10	160	<10	8
		<10	<10	10	<10	80	<10	16
39	27	<10	<10	<10	<10	160	<10	<8
		<10	<10	<10	<10	160	<10	16

lowed to sediment at 37°C. A 1:100 dilution of adenovirus type 6 rabbit serum was used as the diluent in tests involving adenovirus types 1, 2, and 5. Both sera of a pair were always tested at the same time.

Complement-fixation tests were performed as described previously(5) using an antigen prepared from adenovirus type 2 unless otherwise noted.

Results. Each of the pairs of sera was tested for HI antibody against 6 adenovirus serotypes (viz. types 1, 2, 3, 4, 5, and 7) and also for adenovirus CF antibody. The results are shown in Tables 1-6.

Considering first the responses of the children (Tables 1, 2, 3, and 5), a significant (4-fold or greater) homotypic HI antibody rise was found in 24 of the 36 (67%) children studied. If rises from a titer of <10 to 10 are included, 30 of the 36 (83%) children could have been considered to have shown an HI antibody rise to the homotypic virus. Only one child had no homotypic HI antibody in either serum specimen. A relatively low frequency of heterotypic HI rises was noted. Of 168 instances where such an event could have been detected (*i.e.* an HI titer in the first serum of 80 or less) 11 fourfold rises (7%) occurred. Six of these heterotypic reactions were type 1 responses in persons infected with type 5 virus.

Only 11 of 30 children (37%) showed a significant (4-fold or greater) CF antibody response and this low frequency prevailed even among those children with type 2 infections.

The data obtained from adults infected with adenovirus types 4 or 7 (Tables 4 and 6) show that 24 of 27 individuals (89%) showed a significant homotypic HI antibody response and that all could have been considered as showing a homotypic response if rises from <10 to 10 are included. Of the 106 instances where heterotypic HI responses could have been detected 21 rises (20%) actually were observed. Adenovirus types 4 and 7 were the only serotypes isolated from this particular population in the time period studied.

In contrast to the findings among children, 24 of 27 adults (89%) showed a significant CF antibody response. When the 3 individuals who failed to show a significant CF response were tested with CF antigens prepared from the homotypic type of virus, one (GL 500) showed a significant rise and the other 2 did not.

Discussion. These data indicate that the

TABLE IV. Hemagglutination-inhbition and Complement-fixing Antibody Responses of 17 Adults Infected with Adenovirus Type 4

	HI titer with indicated antigen						
Patient	T1	T2	T3	T4	T5	T7	CF titer
GL 222	160	20	160	<10	160	<10	<8
	≧320	≧320	160	≧320	≧320	<10	≧64
224	<10	40	≧320	<10	20	160	64
	160	80	≧320	80	20	80	256
234	10	20	20	<10	10	<10	<8
	40	20	40	80	10	<10	64
241	<10	<10	80	<10	<10	<10	<8
	<10	<10	160	160	<10	<10	≧64
257	80	<10	20	<10	40	<10	<8
	≧320	40	40	80	80	<10	64
489	20	20	160	<10	80	<10	32
	≧320	≧320	160	40	≧320	<10	≧128
497	≧320	160	40	<10	40	10	16
	≧320	≧320	40	80	80	10	64
500	<10	20	10	<10	<10	<10	<8
	40	≧320	20	80	<10	<10	<8
512	≧320	<10	<10	<10	<10	40	16
	≧320	20	<10	40	<10	40	≧128
516	<10	160	<10	<10	80	<10	<8
	10	≧320	<10	10	≧320	<10	128
529	80	≧320	<10	<10	40	<10	<8
	160	≧320	<10	10	40	<10	64
530	10	10	≧320	<10	160	<10	<8
	10	<10	160	40	160	<10	32
573	160	<10	20	<10	80	20	8
	160	<10	20	40	80	20	≧128
574	40	≧320	80	<10	≧320	<10	16
	20	≧320	80	80	160	<10	≧128
585	≧320	<10	40	<10	<10	<10	<8
	≧320	<10	20	40	<10	<10	64
588	≧320	≧320	40	<10	20	40	<8
	≧320	≧320	80	10	20	80	128
597	≧320	20	<10	<10	≧320	<10	<8
	≧320	160	<10	40	≧320	<10	32

HI antibody response to adenovirus infections in man is probably about as specific as that of neutralizing antibody. Thus, in a study(6) of heterotypic neutralizing antibody responses in naval recruits infected with adenovirus types 4 or 7, it was found that significant rises occurred in 60 out of the 240 instances (25%) in which sera were tested against the serotypes used in the present investigation. It is unlikely that the heterotypic responses to types 1, 2, and 5 in either study were due to actual concurrent infection with these viruses since these serotypes are only rarely encountered in adults and were not isolated from any of the subjects studied.

Conversely, types 1, 2, 3, and 5 were quite common at all times in the population from which the children in the present study were drawn(3). Since serum pairs from children often spanned a considerable period of time, it is probable that at least some of the apparent heterotypic responses were actually instances of multiple infection. No heterotypic responses were noted in children to types 4 and 7, which did not occur in the nursery population. Although weekly throat specimens from each child had been tested for adenoviruses, the most sensitive laboratory methods were not always employed and anal specimens were not routinely examined for these viruses. Thus, it was quite possible to have missed the occurrence of a heterotypic infection or to have missed the earliest week of homotypic virus excretion. The latter event is the most likely explanation of those instances in which children were found to have pre-existing homotypic antibody.

While the difference in specificity of HI

TABLE V. Hemagglutination-inhibition and Complement-fixing Antibody Responses of 10 Children Infected with Adenovirus Type 5

Age of child in mo. at time of virus isolation	Days after virus isolation 2nd serum collected	HI titer with indicated antigen T1	T2	T3	T4	T5	T7	CF titer
10	19	<10	<10	<10	<10	<10	<10	≧64
		80	<10	<10	<10	40	<10	128
24	27	80	<10	20	<10	<10	<10	NT
		≧320	<10	<10	<10	80	<10	NT
25	44	<10	160	80	<10	<10	<10	NT
		20	≧320	20	<10	10	<10	NT
26	69	≧320	160	20	<10	<10	<10	NT
		≧320	160	20	<10	40	<10	NT
28	58	40	40	10	<10	<10	<10	NT
		160	80	10	<10	20	<10	NT
29	27	10	80	20	<10	<10	<10	64
		<10	80	20	<10	10	<10	64
29	31	10	80	160	<10	<10	<10	64
		160	≧320	80	<10	20	<10	128
32	43	40	40	80	<10	<10	<10	8
		≧320	40	40	<10	10	<10	32
33	85	40	80	<10	<10	<10	<10	16
		40	80	<10	<10	20	<10	16
37	82	≧320	<10	<10	<10	20	<10	32
		≧320	<10	<10	<10	80	<10	32

TABLE VI. Hemagglutination-inhibition and Complement-fixing Antibody Responses of 10 Adults Infected with Adenovirus Type 7

	HI titer with indicated antigen						
Patient	T1	T2	T3	T4	T5	T7	CF titer
GL 214	≧320	80	<10	≧320	80	<10	256
	≧320	160	<10	80	80	20	256
282	40	80	<10	<10	40	<10	<8
	40	160	<10	40	40	80	<8
291	<10	<10	<10	<10	<10	<10	<8
	10	10	<10	<10	20	80	≧64
350	160	<10	<10	<10	10	<10	<8
	80	<10	<10	<10	10	80	≧64
353	≧320	160	<10	<10	<10	<10	16
	160	160	<10	<10	<10	40	64
490	10	80	10	20	80	<10	8
	10	40	≧320	20	80	≧320	≧128
507	≧320	80	<10	<10	<10	<10	16
	160	80	<10	<10	40	40	128
513	≧320	<10	10	<10	10	<10	16
	≧320	40	≧320	40	10	80	≧128
536	<10	10	<10	<10	<10	<10	<8
	<10	10	<10	<10	<10	20	16
575	160	<10	20	<10	20	<10	8
	160	<10	≧320	<10	20	40	32

antibody responses in children and adults might have been a function of the particular serotypes involved rather than of the age of the subjects, such an explanation is unlikely in view of what is known about serologic responses of children and adults in general. Further study of adenovirus type 3 infections, which occur commonly in both children and adults, should resolve this question.

The CF antibody response appeared to be a quite useful indication of adenovirus infection in adults but was of much less value among children. The latter were more apt to have pre-existing CF antibody, but this factor alone was not responsible for the relatively low rate of significant responses. Thus, of 9 adults with pre-existing CF titers of 8 or 16 all showed significant rises. Of 12 children with the same pre-existing CF titers only 5 (42%) showed significant responses.

Although much remains to be learned about adenovirus HI antibodies such as the time of their first appearance, duration of their persistence, and relationship of heterotypic responses to prior adenovirus infections, the data presently available indicate that they should prove to be useful tools in clinical and epidemiologic studies of adenovirus infections in man.

Summary. Paired sera from 36 children naturally infected with adenovirus types 1, 2, 3, or 5 and from 27 adults naturally infected with adenovirus types 4 or 7 were studied for homotypic and heterotypic hemagglutination-inhibition (HI) antibodies and for complement-fixing (CF) antibody. Homotypic HI responses were observed in most instances. Heterotypic HI responses were relatively uncommon among the children but occurred in adults with about the same frequency as has been reported for heterotypic neutralizing antibody responses in the same type of population. Significant CF antibody responses were detected in most of the adults but in less than one-half of the children.

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Effect of Immune Serum on Hemorrhagic Shock and on Metabolism of Endotoxemia by the R.E. System.[*] (26971)

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The irreversible phase of traumatic shock has been attributed to a loss of the R.E. system's capacity to take up and destroy endotoxin, which is being continuously absorbed into the circulation from the intestine(1). If this is a correct hypothesis, serum containing antibodies to endotoxin might be useful to prevent development of the irreversible phase, because such serum antibodies facilitate uptake of endotoxin by R.E. cells(2).[†] In a normal R.E. cell uptake of enodtoxin is

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[†] This proposal was suggested to us by P. Grabar of Institute Pasteur, Paris.

followed by immediate breakdown(3). In the injured R.E. cell uptake, though greatly reduced, is still appreciable; but significant detoxification cannot be detected(3). From this evidence, one would not expect that serum antibodies would be helpful. Nevertheless, we considered it desirable to investigate the question by direct experiment.

The data reported below demonstrate that serum antibody improves the capacity of the injured R.E. cell to take up endotoxin, but does not restore its capacity to detoxify endotoxin or improve the animal's capacity to tolerate hemorrhagic shock.

Methods and materials. Preparation of immune serum. Serum antibody was produced in 4 ways: 1. Three ml of a heat-killed 18 hour broth culture of *E. coli* 0111B_4 was injected intraperitoneally 3 times a week until the serum agglutination titre reached 1:10,000.‡ This usually required 8 weeks. The animals were rested for 4-5 days and the blood harvested by heart puncture. Injections were then resumed until the next bleed. This routine was maintained with regular checks to determine that the antibody level was still maintained. Sick animals or those showing a drop in titre were discarded.

2. The cells of a heat killed broth culture of the same *E. coli* 0111B_4 were collected, washed 3 times, and suspended in saline to a density of 1500 million/ml. One ml of this suspension was injected intravenously 3 times a week. After a high agglutination titre was reached, the serum was harvested as described.

3. Endotoxin of the same *E. coli* 0111B_4, suspended in saline, was injected intravenously 3 times a week, beginning with a first dose of 0.1 mg followed by graded increases up to 1 mg. After a high agglutination titre was reached (3-6 weeks) the serum was harvested as described.

4. Plasma from rabbits dying of irreversible hemorrhagic shock was injected at regular intervals into the ear vein of normal rabbits. The recipient's serum was harvested when a satisfactory agglutination titre was achieved.

For the study of the capacity of serum antibody to facilitate *in vivo* uptake and destruction of endotoxin, the following experiments were carried out. Dogs were exposed to severe hemorrhagic shock for 2 hours, and were then transfused. One hour later, when systolic blood pressure ranged between 80-130mm/Hg, the peritoneal cavity was opened under pentobarbital anesthesia with sterile precautions, and 2 mg of the *E. coli* endotoxin from *E. coli* 0111B_4 (labelled with P_{32}) in 2 ml of saline was injected during a period of 5 minutes into a cannulated marginal artery of the spleen. Normal or immune serum (18 ml) was injected into the same artery either some minutes before the endotoxin, or with the endotoxin. In some instances the serum and endotoxin were mixed and held at 37°C for 15 minutes before injection. Ten minutes after the injection the spleen was removed and homogenized in normal saline in a sterile endotoxin-free Waring blendor. The splenic homogenate was held at 4°C until examined for toxicity, but P_{32} content was determined at once, and compared with total P_{32} injected, to determine (approximately) the percent uptake of endotoxin(3). Toxicity of the homogenate was determined by the chick embryo method of Smith and Thomas (4). In our experience with this test, if 4 or more of 6 embryos tested are killed, the test substance contains endotoxin; but if 2 or less are killed there is no measurable amount of endotoxin present.

To test the capacity of serum antibodies to prevent development of irreversibility in hemorrhagic shock, the standard 6 hour hemorrhagic shock experiment was carried out in 2 groups of adult albino rabbits:—those with an intestinal flora rich in coliform bacteria, and those with an intestinal flora poor in coliform bacteria. The latter received 2-3 g

TABLE I. Effect of Immune Serum on Survival Rate of Rabbits Exposed to Hemorrhagic Shock for 6 Hours.

Type of intestinal flora	No. exp.	Immune serum	Survival rate in %
Coliform rich	14	—	7
" "	11	+	9
Coliform-poor plus *E. coli* by gavage	6	—	0
idem	13	+	0

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‡ By the Widal tube dilution technic.

TABLE II. Effect of Immune Sera on Uptake and Detoxification of Endotoxin by Injured Spleen.

No.	% Uptake by spleen		Toxicity of homogenized spleen	
	Normal serum	Immune serum	Normal serum	Immune serum
A. Infusion of Serum 10 min. Before Endotoxin				
1	14	33(1:640)*	+ (5/6)**	— (1/6)**
2	20	13(1:2560)	+ (5/6)	+ (5/6)
3	19	37(1:2560)	+ (6/6)	? (3/6)
4	4	35(1:2560)	+ (6/6)	+ (6/6)
5	5	14(1:1280)	? (3/6)	? (3/6)
6	4	17(1:640)	+ (6/6)	+ (6/6)
7	15	11.5(1:10,000)	+ (5/6)	+ (6/6)
Avg.	11.5%	22.9%		
B. Infusion of Mixture of Immune Serum and Endotoxin				
1	33	31(1:10,000)	+ (6/6)	+ (6/6)
2	12	54(1:10,000)	+ (6/6)	+ (6/6)
3	10	47(1:10,000)	+ (6/6)	— (1/6)
4	14	48(1:10,000)	+ (6/6)	? (3/6)
5	—	20(1:10,000)	—	+ (6/6)
6	—	39(1:10,000)	—	+ (6/6)
7	—	20(1:10,000)	—	+ (4/6)
8	—	40(1:10,000)	—	+ (6/6)
Avg.	17.2%	37.4%		

* Figures in parentheses show agglutination titre of immune sera.

** Figures in parentheses show mortality of embryos tested for presence of endotoxin; 4 or more per 6 tested is considered positive; 3 of 6 is borderline; and less than 3 is considered negative.

(wet wt) of *E. coli* 0111B$_4$ by gavage 3-4 hours before shock was started. Twenty to 30 ml of immune serum was given intravenously 18 hours, 2 hours, 30, 15 or 5 minutes before starting the hemorrhage, or 15 to 30 minutes after starting the hemorrhage. Because none of the immune sera were specific for the endotoxins produced by the normal intestinal flora of the rabbit, it was expected that their potential effectiveness would best be evoked in the coliform-poor rabbits gavaged with the *E. coli* 0111B$_4$.

Results (Tables 1 and 2). Table 1 shows the effect of immune serum on survival rate after exposure to 6 hours of hemorrhagic shock. Of the series of 44 rabbits, approximately half received no serum or normal serum, the remainder immune serum, in most instances within 30 minutes prior to shock. The immune serum produced no change in survival rate or the pathology at death.

Table 2 shows the effect of various types of immune serum on uptake and detoxification of endotoxin by the injured spleen. The difference in uptake between shocked spleens pretreated with normal and immune sera is enough to indicate a favorable effect of the serum antibodies on uptake of endotoxin.

The degree of detoxification achieved by the shocked spleens was assayed in terms of percent of surviving chick embryos. The effect of immune serum so determined does not indicate a significant improvement in anti-endotoxic action of the R.E. cell.

Summary and conclusion. Serum containing antibody specific for an endotoxin improves uptake of the endotoxin by the damaged R.E. system. But breakdown of the endotoxin taken up by this system is not thereby improved. Such immune sera given to rabbits in severe hemorrhagic shock for the purpose of improving the clearance of circulating endotoxin do not lower the mortality rate.

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Effect of Inhibiting both Catechol-O-Methyl Transferase and Monoamine Oxidase on Cardiovascular Responses to Norepinephrine. (26972)

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Norepinephrine is metabolized *in vivo* largely by 2 enzymes—catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO)(1). It should not be assumed, however, that metabolic destruction of norepinephrine by these enzymes is necessarily the major process by which the amine is inactivated pharmacologically. There is some evidence, in fact, that MAO may not participate in terminating the cardio-vascular actions of norepinephrine administered intravenously in the dog; complete inhibition of MAO in this species fails to potentiate the effects of norepinephrine(2). The following study was designed to obtain comparable data on the effect of inhibiting COMT alone and of inhibiting both enzymes simultaneously. This report describes the effect of enzyme inhibition on the cardio-vascular actions of norepinephrine and on the disappearance of norepinephrine from the circulation after its intravenous injection in the dog.

Methods. Studies were performed in open-chest, vagotomized dogs (13-18 kg) anesthetized with a combination of pentobarbital and barbital. Arterial pressure and cardiac contractile force (measured by a strain gauge arch sutured to the right ventricle) were monitored continously as described previously(2). A control dose of *l*-norepinephrine (1-3 μg/kg) was given intravenously into a femoral vein, and blood pressure and heart force responses were recorded. A potent MAO inhibitor, 1-phenyl-3-hydrazinobutane (JB-835), was then administered intravenously in a dose of 5 mg/kg. After 60-90 minutes had elapsed, 2 intravenous doses of pyrogallol (each 37.5 mg/kg) were given 15 minutes apart in order to inhibit COMT. Five to 10 minutes later the pretreatment dose of *l*-norepinephrine was repeated, and cardio-vascular responses were again recorded. After each injection of norepinephrine, blood samples were drawn at 1-5 minute intervals from a femoral artery; plasma norepinephrine concentration was determined in each sample by a fluorometric method(3). In preliminary studies on the effect of COMT inhibition alone, administration of JB-835 and determination of plasma norepinephrine levels were omitted; the procedure was the same in all other respects. At the end of each experiment COMT activity was determined(4) in the myocardium and liver.

Results. Effect of pyrogallol on cardiovascular actions of l-norepinephrine. Preliminary observations were made in 2 dogs treated only with the COMT inhibitor. After injection of pyrogallol the cardiac contractile force increased slowly and progressively; blood pressure also increased, but to a lesser degree. This response to certain polyphenols, among them pyrogallol, has been shown to be due to central nervous system stimulation(5). Since blood pressure and heart force remained near control levels for only about 15 minutes after the second dose of pyrogallol, the study was limited to a comparison of single injections of *l*-norepinephrine before and after COMT inhibition. There was no potentiation of the peak response of the blood pressure or cardiac contractile force to injected norepinephrine (1-2 μg/kg); however, return of these parameters to baseline values was prolonged somewhat after enzyme inhibition. COMT activity was found to be inhibited 100% in the myocardium and 92-100% in the liver by these doses of pyrogallol.

Effect of pyrogallol plus JB-835 on cardiovascular actions of norepinephrine. Since marked potentiation of the action of norepinephrine did not occur after COMT inhibition alone, the experiment was repeated in 3 dogs treated with a MAO inhibitor (JB-835) in addition to pyrogallol. Combined treatment with pyrogallol and JB-835 produced no augmentation of the peak responses to injected norepinephrine (Fig. 1). However,

TABLE I. Prolongation of Cardio-vascular Responses to L-norepinephrine in the Dog by Combined Administration of JB-835 and Pyrogallol.

		Time from onset of response to complete return to baseline value					
		Blood Pressure			Cardiac Contractile Force		
Dog	Dose of L-norepinephrine (µg/kg)	Before* (sec.)	After† (sec.)	% Increase	Before (sec.)	After (sec.)	% Increase
A	3	150	295	97%	180	320	78%
B	2	200	325	63	160	240	50
C	1	140	140	0	140	155	11

* Before = before administration of enzyme inhibitors (control injection).
† After = after administration of enzyme inhibitors (see text for dosages).

the time required for blood pressure and cardiac contractile force to return completely to baseline was moderately prolonged (Table 1); degree of prolongation increased as dosage of norepinephrine was increased. In addition, the inhibition of these enzymes delayed the disappearance of norepinephrine from the arterial plasma (Fig. 2). This delay was present at all times after the injection, but was most pronounced, relative to the control injection, during the latter phases of the disappearance curve (between 2½ and 10 minutes after injection).

Discussion. These results indicate that the cardio-vascular actions of injected norepinephrine can be terminated in the dog in the functional absence of both enzymes which metabolize the amine. The pharmacological inactivation of circulating norepinephrine under these conditions occurs more slowly than normal. Part (and perhaps all) of this prolongation of action can be attributed to a delayed disappearance of norepinephrine from the blood stream, probably due to decreased destruction of circulating amine by the liver and kidney(3). The failure of combined COMT and MAO inhibition to potentiate markedly the cardio-vascular effects of injected norepinephrine suggests that pharmacological inactivation of circulating norepinephrine does not require the intact function of either enzyme at the receptor sites. This conclusion may not necessarily apply to norepinephrine released directly into the tissues by the sympathetic nerve endings.

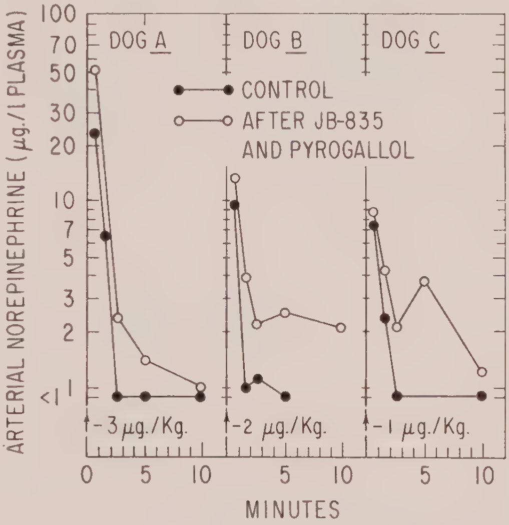

FIG. 2. Concentration of norepinephrine in arterial plasma after single intravenous injections of *l*-norepinephrine in 3 dogs: Before and after simultaneous administration of JB-835 and pyrogallol.

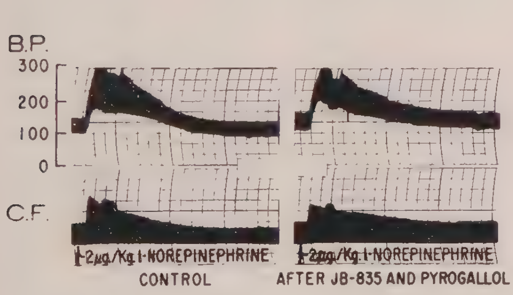

FIG. 1. Effect of JB-835 and pyrogallol on cardiovascular responses to *l*-norepinephrine (Dog B of Table 1 and Fig. 2). B. P. = blood pressure; C. F. = cardiac contractile force. Each thin vertical line represents 20 sec.

Dilution and redistribution, as well as metabolic destruction, undoubtedly contribute to the rapid decline in the norepinephrine concentration of the plasma after single intravenous injections of the amine. However, complete equilibration of norepinephrine with total body water would have to occur within a period of 2-2½ minutes to account for the plasma clearance curves of Fig. 2 solely on the basis of redistribution. This rapid rate of clearance, even after the inhibition of amine-metabolizing enzymes, is compatible with the observation that circulating catecholamines are taken up quickly by many tissues of the body(3,6).

Summary. The simultaneous inhibition of catechol-O-methyl transferase and monoamine oxidase (by pyrogallol and JB-835) produced only moderate prolongation of the cardiovascular effects of injected *l*-norepinephrine in anesthetized dogs. This prolongation of pharmacological action in presence of enzyme inhibitors was correlated with a delayed disappearance of injected norepinephrine from the circulation. While these results indicate that metabolism by these enzymes contributes to removal of circulating norepinephrine from the plasma in the dog, they further suggest that the physiological inactivation of circulating norepinephrine at the receptor sites does not require metabolic destruction of the amine by these enzymes.

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Thyroid: Serum Radioiodide Concentration Ratio During the Estrous Cycle of the Mouse.* (26973)

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The thyroid gland is known to exhibit variations in its activity which are closely correlated with changes in the estrous cycle. The thyroid concentrates more iodide during late estrus in rats(1) and in estrus in rabbits(2). However, in mice it has been reported to be more active during proestrus(3). Because of this apparent species difference it was of interest to reinvestigate thyroid activity in cycling mice. In this investigation thyroid: serum radioiodide concentration ratios (T/S) were determined simultaneously with the identification of the stage of the cycle. This measurement is much less influenced by factors outside the pituitary-thyroid axis than is percentage uptake of I^{131} as has been used by previous investigators. Also, these workers have determined uptakes on the basis of 4 stages of the estrous cycle; it was felt that by subdividing the cycle into 6 stages, more precise information could be obtained concerning the relationship between thyroid function and the reproductive cycle of mice.

Material and methods. Adult Swiss Webster mice weighing between 25 and 30 g were used in this experiment. All were fed Rockland laboratory chow *ad libitum* for at least one week before measurement of the T/S.

To determine T/S, 1 mg of propylthiouracil (PTU) in 0.25 ml of alkaline solution was injected subcutaneously 45 minutes before a subcutaneous injection of 1 μc of carrier-free I^{131} in 0.25 ml of distilled water. One hour later, the animals were anesthetized with ether and blood was obtained from the abdominal aorta. The thyroid was dissected out and weighed on a Roller-Smith torsion balance. One-tenth ml of serum and the thyroid were prepared for radioactivity determination by the method described by VanderLaan and Greer(4).

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* Supported by USPHS grant #A-4129.

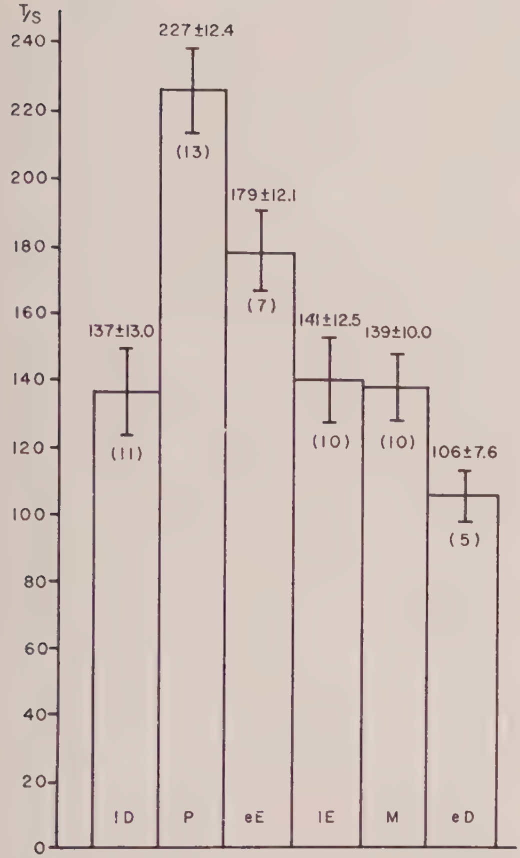

FIG. 1. Thyroid : serum radioiodide concentration ratios (T/S) during the various stages of the estrous cycle. Vertical bars represent mean T/S values, vertical lines standard errors of means. Figures within bars represent number of mice in group. (P = proestrus, eE = early estrus, lE = late estrus, M = metestrus, eD = first half of diestrus, lD = second half of diestrus).

P values:

P vs eE	.05-.02	eE vs lE	.05	lE vs eD	n.s.
P vs lE	<.01	eE vs M	.05-.02	lE vs lD	n.s.
P vs M	<.01	eE vs eD	<.01	M vs eD	<.05
P vs eD	<.01	eE vs lD	.05-.02	M vs lD	n.s.
P vs lD	<.01	lE vs M	n.s.	eD vs lD	n.s.

Vaginal smears were taken for 5 or more days and only animals that were cycling were used. Since the diestrus interval is commonly the longest interval and the most variable, we found it convenient to divide it into a first and second half. First half of diestrus was considered to be the day following metestrus, whereas the second half was the second day of diestrus. On the day of the experiment vaginal smears were performed approximately one to 5 minutes before the animal was sacrificed. Only those mice whose vaginal smears could be identified unequivocally as typical of one of the 5 stages of the estrous cycle (proestrus, early estrus, late estrus, metestrus, and diestrus) were used in the experiment.

Results. As shown in Fig. 1, the thyroid: serum radioiodide concentration ratio was found to be highest during proestrus. This value was significantly different from all other stages. With the onset of early estrus T/S decreased, reaching its lowest level in the first half of diestrus. T/S ratios of late estrus, metestrus, and second half of diestrus were essentially alike and not significantly different from the values found during the first half of diestrus.

Discussion. Our results confirm the finding of Soliman and Reineke(3) that in cycling mice the maximum uptake of radioactive iodide is obtained during proestrus. However, by dividing the estrous cycle into 6 stages, we were able to demonstrate that the thyroid also concentrates a significantly greater amount of radioiodide during early estrus than during the other stages of the cycle. Furthermore, we did not find an increased incorporation of radioiodide in diestrus as did Soliman and Reineke(3). In fact, the first half of diestrus was found to be the least active and the second half of diestrus was essentially the same as late estrus and metestrus. The explanation for the difference between the 2 findings may be due to their determining uptakes 6 hours after identification of the stage of the estrous cycle. It is conceivable that some of the animals were actually in proestrus at time of sacrifice, which then resulted in finding false high values for the diestrous period.

The thyroid:serum radioiodide concentration ratio of cycling female rats is at its lowest level during proestrus and highest during late estrus(1). This is in direct contrast to the present findings in the mouse. It appears that the increased T/S ratios in cycling animals may be due to a rise in blood levels of estrogen, since the exogenous administration of this hormone has been reported to increase I^{131} uptake(5,6) and T/S ratios (unpublished data). Also, Moon and Turner(7) have shown a reduction in thyroid function following ovariectomy and that estradiol prevents the decrease in thyroid secretion rate. Ovulation in both species is different in regard to its occurrence in the estrous cycle. In Wistar rats it occurs some 9-12 hours after onset of estrus(8). However, in albino mice it usually occurs about 2 hours after onset of estrus and sometimes earlier(9). Consequently, estrogen secretion probably occurs sooner and reaches a maximum concentration earlier in the mouse than in the rat. Therefore, the difference between the 2 species may be due to: 1) the release and/or increased concentration of estrogen occurring sooner in mice; or 2) a difference in thyroidal, or hypophyseal, or hypothalamic sensitivity to blood levels of estrogen.

The measurement of thyroid:serum iodide gradient is a very sensitive index of the level of circulating thyrotropic hormone(4,10). Therefore, it appears from our data that the maximum amount of TSH occurs during proestrus and the least amount during the first half of diestrus. However, in hypophysectomized rats injected with a single dose of TSH, the T/S does not increase for at least 6 hours. The maximal effect on the iodide pump occurs 1-2 days after injection of TSH (10,4). Hence the change in thyroid function during any one of the stages of the estrous cycle may in fact have been initiated during an earlier stage, since a transitory depression of T/S preceding its rise has been found to occur after injection of TSH into hypophysectomized rats(11). Another possibility is that TSH affects T/S at least in part through alterations of the organic iodine concentration in the thyroid(12). However, it is not known whether a substantial depletion of organic thyroidal iodine occurs during estrus, and if so whether such a change affects the T/S promptly.

Summary. The thyroid:serum radioiodide concentration ratio (T/S) was investigated in mice during the different stages of the estrous cycle. T/S was found to be at its lowest levels during diestrus and highest during proestrus. T/S values during the other stages of the cycle were intermediate and not significantly different from each other. The results indicate that the ability of the thyroid to concentrate iodide from the serum shows cyclic changes which can be correlated with stages of the estrous cycle. Also, the period of maximal thyroid activity is different from that previously noted in rats.

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Immune Response in Irradiated Mice with Peyer's Patch Shielding.* (26974)

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We reported(1) that the capacity to produce antibodies to an injected particulate antigen was retained in the rabbit if the spleen or appendix were shielded while the rest of the body received an LD_{50} dose of x-radiation. We were able to demonstrate that this capacity was retained if these shielded tissues were removed 24 hours, or even as soon as 3 hours, after irradiation(2). These observations have been confirmed by Taliaferro and others(3,4). We also showed that a more rapid regeneration of the lymphatic tissues occurred in rabbits which had the appendix or spleen shielded during irradiation than in control animals. These studies were conducted using dosages of irradiation in the LD_{50} range, thus permitting the bone marrow of survivors to regenerate, though not as rapidly as the lymphatic tissues(5). Recently Süssdorf(6) has repeated our original study and found an essentially similar course of events.

One of our most interesting findings was that no granulocytopoiesis, megakaryocytopoiesis or erythropoiesis was observed in the shielded appendix, suggesting that under the conditions of the experiment these lymphatic cells did not exhibit a multipotential capacity as far as local production of other blood elements was concerned. In the light of present knowledge concerning cellular colonization(7,8,9), the rapid proliferation of lymphatic tissues following appendix- or spleen-shielding is presumably the result of migration and subsequent colonization of cells from the protected lymphatic tissues.

It seemed of interest, therefore, to repeat the experiment of protecting a pure source of lymphoid cells during total-body exposure to a supralethal dose of x-radiation, and to study 1) lymphocyte migration, recolonization, and potential for the production of other cell types; 2) modifications of the immune response that might result; and 3) value of the technique as an assay of the control of lymphopoiesis.

A patch of Peyer in the small intestine of the mouse measuring about 2.5 mm in width and 1 mm in depth and weighing about 5 mg was selected for these studies. Approximately 5 million fixed and free lymphatic cells are contained in such a patch as estimated by geometric calculations.

Adult female CF No. 1 and (C3H × $101)F_1$ mice approximately 14 weeks old, were used as recipients in most of these studies. Donor tissues were obtained from female A/Tex mice and from male Holtzman rats.

The shield used to protect the Peyer's patch during irradiation was constructed of ¼ inch lead, 11 mm wide, with a groove 4 mm in diameter to house the intestine. Under Nembutal anesthesia, the small intestine was brought out through a small incision into the peritoneal cavity and a single Peyer's patch was shielded during total-body irradiation with 950 r. In control groups, either an equivalent portion of the intestine having no Peyer's patch was shielded, or a sham operation was performed without any shielding. Following irradiation the intestine was returned to the peritoneal cavity and the incision sutured with silk.

Since 100×10^6 rat bone marrow or 10×10^6 mouse bone marrow cells afford good initial protection in lethally irradiated mice, these cell concentrations were used in the experiment. The technique for preparation of the various suspensions has been described (10). The suspensions were made in iso-

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‡ Dept. of Med., The Univ. of Chicago.

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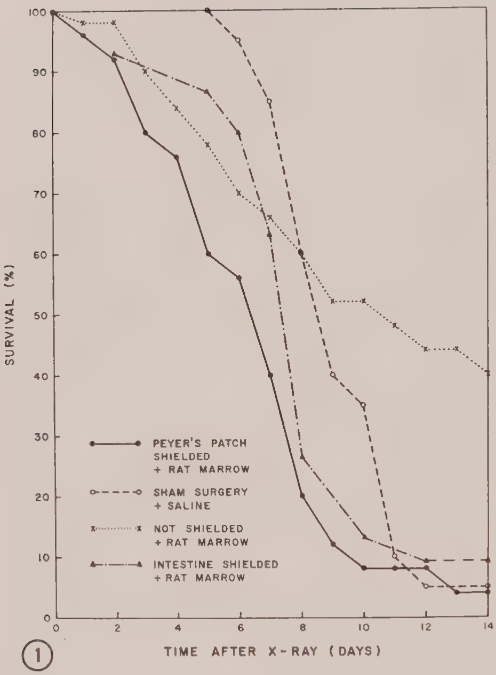

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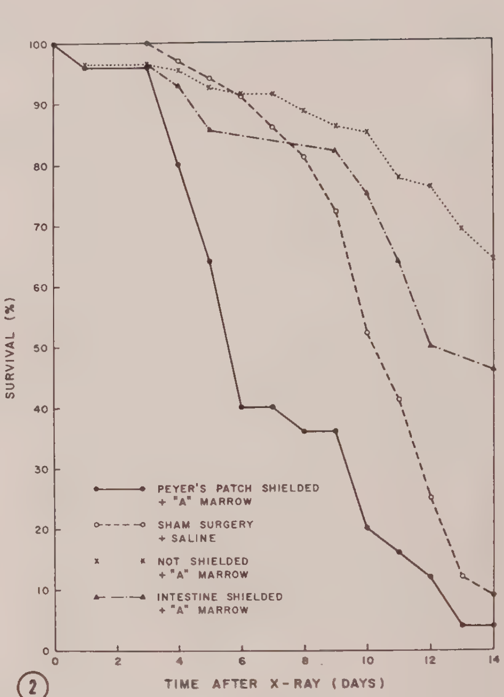

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FIG. 1. Survival of CF No. 1 mice receiving 950 r and treatments as shown.

FIG. 2. Survival of (C3H × 101)F_1 mice receiving 950 r and treatments as shown.

tonic saline and 0.5 ml injections were given via the tail vein.

Dosimetry. The x-rays were generated in a 250 kv G.E. Maxitron operating at 30 Ma and using 0.25 mm copper and a 1-mm aluminum filter. The half-value layer in copper of the filtered beam was 1.02 mm. The exposures were measured with a Victoreen condenser meter equipped with a 250 r chamber. Measurements were made in air at a position estimated to be the center of the animal's body. The dose rate averaged 120 r per minute at 57 cm.

Results. Survival. As can be seen in Fig. 1, 40% (20 of 50) CF No. 1 mice in the control group given rat bone marrow following total-body irradiation without shielding were alive at 14 days, whereas only 4% (1 of 25) survived in the group given rat bone marrow following protection of one Peyer's patch. Furthermore, the survival pattern shows that death in such Peyer's patch protected mice occurred significantly earlier ($P<0.001$) than in sham operated controls that were lethally irradiated without shielding and without cellular injection.

In an additional control group, an 11-mm portion of intestine that contained no Peyer's patch was protected during exposure to 950 r, and these mice were also injected with rat bone marrow cells. Death also occurred significantly earlier in this group ($P<0.02$) than in irradiation-only controls, but the results were not as dramatic as in the Peyer's patch group. By the 14th day, however, only 9% (2 of 22) were alive.

In a companion series of experiments using homologous strains, when (C3H × 101)F_1 hybrid mice were given bone marrow from Strain A/Tex mice following Peyer's patch shielding during irradiation with 950 r, an early death pattern also resulted (Fig. 2). In this strain combination, however, protection of 11-mm of intestine lacking a Peyer's patch did not produce an equally severe reaction, and 48% (14 of 29) were alive at 14 days. This may be interpreted as indicating that fewer lymphoid cells are required to re-

TABLE I. Comparative Effect of Varying X-ray Dosages Delivered to Shielded Peyer's Patch and Intestine on Lethally Irradiated (950 r) CF No. 1 Mice Injected with Rat Bone Marrow.

X-ray dosage to Peyer's patch (r)	No. of mice used	14-day no.	Survival (%)	X-ray dosage to 1.1 mm intestine	No. of mice used	14-day no.	Survival (%)
None	25	1	4	None	15	1	6.6
200	10	0	0	200	10	0	0
300	10	0	0	300	10	0	0
400	10	0	0	400	10	2	20
600	10	0	0	600	10	1	10
650	10	3	33.3	650	10	4	40
700	10	6	60.0	700	10	9	90
950	50	20	40.0	950	50	20	40

ject a heterologous graft than to reject an homologous graft.

Furthermore, it must be kept in mind that in addition to the fact that the genetic relationship in this experiment is closer than in the rat-to-mouse combination (thus diminishing the antigenic differences), only 10 × 10^6 mouse bone marrow cells were injected while 100 × 10^6 rat cells were used. In a preliminary experiment, (C3H × 101)F_1 mice that received larger numbers of A/Tex bone marrow cells (20 to 40 × 10^6) following the shielding of 11 mm of the small intestine did not survive.

These results led us to investigate whether the shielding effect could be reproduced by intravenous injection of isologous lymphocytes or spleen cells into mice treated with rat bone marrow following exposure of the entire body to lethal irradiation. Lymphocytes from the mesenteric lymph node and spleen cells from adult CF No. 1 donors were injected into CF No. 1 mice 4 hours after the irradiation bone-marrow treatment. Results agree with those of van Bekkum and Vos(11) and indicate that in the CF No. 1 mouse also, fewer than one million cells from lymphatic tissue or spleen of its own strain, given by intravenous injection, hasten the death of the recipient.

Hybrid (C3H × 101)F_1 mice that received A/Tex bone marrow (10 × 10^6 cells) or rat bone marrow (100 × 10^6 cells) after total body x-irradiation and were subsequently injected with 10 × 10^6 cells from the mesenteric lymph node of their own strain responded in the same manner, and all mice died by the 14th day after treatment.

It requires a supralethal dose of whole-body x-irradiation (about 900 r) to suppress the capacity of the mouse to reject a heterologous (rat) bone marrow graft. The amount of x-irradiation required to suppress the capacity of the Peyer's patch or of 11 mm of intestine to reject a heterologous graft, was determined by removing the lid from the shield after a calculated number of roentgen units had been given to the mouse. Thus the protected Peyer's patch or intestine received dosages of from 200 r to 700 r at the same time that the rest of the body received 950 r (Table I). These values were found to be 650 r and 400 r respectively, and suggest that the smaller the lymphoid population, the smaller is the dose of x-irradiation necessary to suppress the total immune response. These findings also indicate how few immunologically competent cells need remain in the body after irradiation successfully to defeat heterotransplantation.

In another series of experiments, the Peyer's patch was protected initially while the body was given 950 r. Within 10 minutes the protected patches were then given 950 r, followed by injection of 100 × 10^6 rat bone marrow cells. All mice died and the early death pattern was again observed, suggesting that following irradiation stress there is immediate migration of lymphoid cells from the Peyer's patch into the damaged body, where they presumably recolonize and maintain the immune response of the individual.

Histology. Histological examination of mice following Peyer's patch protection shows that before death the shielded patch has accomplished partial or essentially complete re-

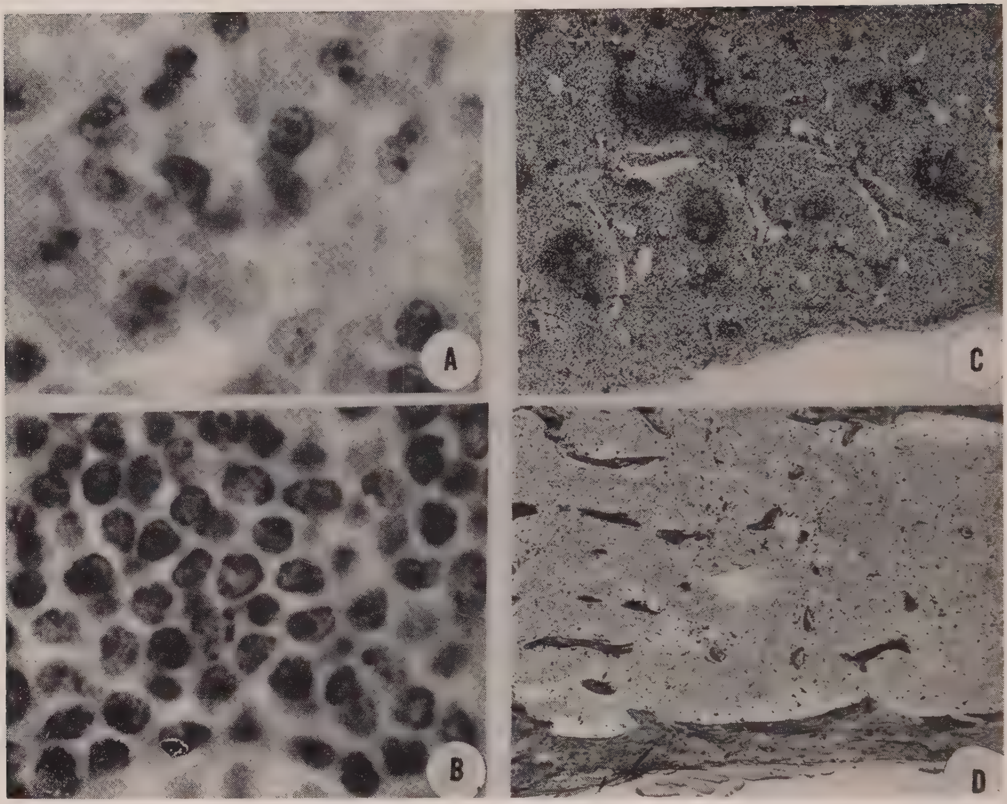

FIG. 3. A—Mesenteric lymph node of CF No. 1 mouse 6 days after 950 r with Peyer's patch shielded, showing plasma-like cells (× 1567). B—Normal mesenteric lymph node of CF No. 1 mouse (× 1567). C—Spleen of CF No. 1 mouse 6 days after 950 r with Peyer's patch shielded, showing foci of lymphoid recolonization (× 36). D—Aplastic bone marrow in CF No. 1 mouse 6 days after 950 r with Peyer's patch shielded, showing absence of any recolonization (× 36).

population of the depleted lymphatic structures, including the thymus. We have sacrificed such animals at daily intervals from 1 through 12 days after the shielding-irradiation procedure. Celloidin-embedded tissues were cut at 6 μ and sections stained with hematoxylin-eosin and azure. In the shielded Peyer's patch itself we observed increased mitotic activity 24 hours after shielding, and this may be seen in other lymphoid structures as repopulation proceeds. In many animals repopulation of the spleen, lymph nodes, and thymus is well along at 6 days, but essentially complete repopulation is not accomplished before 10 to 12 days (Fig. 3). No bone marrow regeneration was observed (Fig. 3-D) nor was there evidence of granulocytic, megakaryocytic, or erythrocytic regeneration elsewhere.

One interesting finding in these mice, for which we have no explanation, is the great prominence of plasma (or plasma-like) cells in the regional mesenteric lymph node during repopulation (Fig. 3A). These plasma cells, which by 3 days constitute about 90% of the free cell population at this site, are found principally in association with the blood vessels. They show marked mitotic activity, and under low power light microscopy the lymph node appears to be composed of virtually hundreds of rosettes of them.

An increased number of such plasma (or plasma-like) cells is also found in the mesenteric node of mice given whole-body x-radiation in the LD_{50} range or above, without Peyer's patch shielding, but in this case not until 6 to 8 days postirradiation. Since these cells are morphologically identical in both experimental models, we made an attempt to determine whether they were also function-

Table II. Effect of Lymph Node Transplantation on Survival of 950 r Irradiated CF No. 1 Mice Treated with Rat Bone Marrow.

Group	Donor mesenteric* lymph nodes	Survival (days)	
		8	14
1	2 nodes from mice 6 days after 950 r	17/18	14/18
2	2 nodes from mice with shielded Peyer's patch 6 days after 950 r	3/14	2/14
3	One-half normal node	2/24	0/24
	One-fourth " "	7/11	1/11

* Transplantation of spleens instead of lymph nodes from similarly treated mice gave comparable results.

ally identical in both situations, and the following experiment was performed to test the capacity of a mesenteric node transplant to reject a rat bone marrow graft previously given to irradiated mice.

CF No. 1 mice were lethally irradiated and treated with 100 × 10^6 rat bone marrow cells. About 3 hours later, 2 mesenteric lymph nodes taken from mice 6 days after exposure to 950 r were transplanted to the peritoneal cavity of one group of recipients (Table II). Similarly, 2 mesenteric lymph nodes obtained 6 days after lethal irradiation from mice having a shielded Peyer's patch were implanted into another group of rat-treated irradiated mice. As a control procedure, one-fourth to one-half of a normal lymph node was transplanted to a third group of irradiated mice treated with rat bone marrow. Average weights of the mesenteric lymph nodes used were: Normal mice, 110 mg; 950 r x-irradiated mice, 42 mg; irradiated, Peyer's patch shielded mice, 53 mg.

The data in Table II indicate that immunologically competent cells were present in the mesenteric lymph node 6 days after mice were lethally irradiated with the Peyer's patch shielded. The nodes from irradiated mice without such shielding either contained no immunologically competent cells or contained a number insufficient to reject the heterologous graft. Transplantation of a normal lymph node caused rejection of the rat bone marrow group in the control animals.

These results were not unexpected since it is well known(11) that cells from a normal mesenteric lymph node transplant will reject a rat bone marrow graft previously given to lethally irradiated mice. Neither was it unexpected that the mesenteric node from a mouse previously given whole-body x-radiation should fail to cause rejection of a rat bone marrow graft which had been given to an irradiated mouse. However, the mesenteric node from Peyer's patch-shielded mice proved as capable of rejecting a rat bone marrow graft as the normal node. This latter observation indicates that although these 2 groups of nodes are rich in plasma cells that appear to be morphologically alike, they are functionally quite different. To be sure, the mesenteric nodes in both these groups contained perhaps 10% lymphocytes, or lymphocyte-like cells, so one cannot be sure that the plasma cells were responsible for the immune response. It can be stated categorically, however, that even though the population of these 2 experimental groups is morphologically similar, their functional capacities are vastly different.

Discussion. It is now well established that lethal irradiation destroys the ability of the mouse to react against foreign bone marrow cells(7,8,9), and that such injected cells colonize and substantially reduce acute radiation mortality. In Peyer's patch-shielded animals injected with heterologous or homologous bone marrow cells, the grafted cells are rejected by the mouse's own regenerating lymphatic tissues, and since lymphocytes alone cannot support life at 950 r, the mice succumb. Lymphoid repopulation is well established in the spleen and mesenteric lymph node by the sixth day, and this coincides with the early death pattern observed. Histological evidence shows that lymphocytes normally tend to accumulate in the intestinal wall. Even though it is doubtful that as many as 5 × 10^6 are present in an 11-mm segment, the supply of lymphatic elements made available by shielding is sufficient to elicit rejection of the foreign rat cells. The results obtained when a Peyer's patch is shielded during lethal irradiation and the host is subsequently injected with heterologous cells agree with those of van Bekkum and Vos(11). These investigators treated irradiated mice with rat bone marrow and subsequently injected them with a small number of isologous lymph node cells, thus preventing the transplant of the rat bone marrow from being successful. Barnes(12) reported similar results in irradiated mice treated with homologous marrow before isologous lymph node cells were injected. We, too, have found that relatively few cells from lymphatic tissues are needed to reject a heterologous or homologous graft.

The lymphocytic tissue in the Peyer's patch of the guinea pig are reportedly at least as active, if not more so, than those in the mesenteric lymph nodes. The germinal centers tend to be larger in the Peyer's patches than in the lymph nodes, while mitoses are especially numerous in the peripheral part of the center(13).

Cole and Garver(14) report that intraperitoneal injection of 0.6 cc of mouse blood (either isologous or homologous) annuls the protective effect of rat bone marrow in LAF_1 mice. These authors indicate that when the blood and marrow are of the same strain (C3H) there is no indication that the C3H bone marrow transplant is rejected.

Goodman and Congdon(15) reported the "killing-effect" in irradiated mice of a mixture of homologous bone marrow and isologous blood. Histologic studies showed evidence of temporary transplantation of bone marrow and then rejection. Irradiation of the blood, or of the donor prevented this reaction. These results support the theory that immunological reactive cells are present in the peripheral blood.

Santos and Cole(16) have also shown that injection of isologous lymphoid tissue from the spleen or lymph thymus elicits early rejection of rat marrow transplants in irradiated mice.

Under the experimental conditions described, the lymphoid cells of the Peyer's patch, although capable of repopulating other lymph structures, cannot serve as precursors of other cell types of the blood-forming tissues.

It is interesting to note that an x-ray dose of 650 r or more is required before the capacity of the lymphatic tissue in the Peyer's patch or intestine is impaired sufficiently to permit retention of a heterologous graft.

This model, which involves survival of tissue foci *in vivo,* provides us with an opportunity of studying the factors involved in normal control of growth and differentiation of lymphoid tissue, as well as of evaluating the substances which will enhance or inhibit immune responses.

Summary. 1. Shielding one Peyer's patch of a mouse during lethal x-irradiation protects a sufficient number of cells to cause rejection of a heterologous or homologous bone marrow graft. 2. Shielding 11 mm of the small intestine results in a similar but less violent reaction. 3. A dosage of 650 r or more to the protected Peyer's patch is necessary to impair the capacity of the lymphatic tissue to reject the graft. 4. Regeneration of lymphatic tissue is evident in the spleen, mesenteric lymph node and thymus in Peyer's patch-shielded mice 6 days post-irradiation. On the other hand no bone marrow regeneration was observed nor was there evidence of granulocytic, megakaryocytic, or erythrocytic regeneration elsewhere.

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Effect of Phosphate Loading on Leukocyte Labelling with Inorganic P^{32}.* (26975)

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Deoxyribonucleic acid (DNA) labelling with inorganic radiophosphorus (P^{32}) has been used to study leukocyte kinetics (1-11); the usefulness and reliability of this leukocyte label for appraising cell kinetics has been considered previously(12, 13). It has been shown that isotopic labelling of DNA occurs only during mitotic interphase when new DNA is synthesized(1,2,14-16) and that DNA retains label introduced during synthesis until cell death ensues(1,3,4,16-18).

Previous studies in rabbits(13) demonstrated that significant levels of plasma phosphate activity persist for at least 7 days after a single intravenous injection of only 2 μc of inorganic P^{32}/kg. Ultimate distribution of P^{32} to reach a common level of specific activity of the phosphate in the crude acid soluble, phospholipid, RNA, and DNA fractions of marrow cells was observed to occur by 72 hours. At this time the curve of regression of plasma phosphate activity, comparable in level to that of cell fraction phosphate, was noted to be quite flat. It was concluded that significant DNA labelling must continue for several days following a single intravenous dose of P^{32}. In an effort to shorten the exposure time of proliferating cells to high levels of activity in the extracellular phosphate pool, a load of non-isotopic inorganic phosphate was given intravenously and its effect on plasma clearance of P^{32} and on cell labelling evaluated.

Materials and methods. These studies were done in 3-5 kg rabbits each of which received intravenously 2.0 μc of P^{32}/kg of body weight. Technical and analytical procedures have been described(12,13). Serial blood samples to evaluate plasma clearance following intravenous P^{32} were obtained from the superior vena caval system via a size PE-50 polyethylene catheter inserted through a marginal ear vein. Samples showing visible hemolysis were discarded. Plasma was treated with an equal volume of 10% trichloroacetic acid and the supernate obtained after centrifugation was wet ashed in concentrated sulfuric acid prior to phosphorus determination.

Leukocytes were obtained from heparinized blood by the method of Athens, *et al.*(19). Red cells were sedimented in dextran and those remaining in the leukocyte containing supernate were hemolyzed with gramicidin-lysolecithin. Leukocytes were washed in saline and platelets removed by differential centrifugation. The remaining leukocyte suspension was then fractionated by a modification of the Schmidt-Thannhauser procedure, quantitative phosphorus analysis performed by an adaptation of the method of Berenblum and Chain, and radioactivity determined by scintillation counting(12).

Non-isotopic inorganic phosphate was given as a sterile, pyrogen-free solution containing approximately 100 millimols of phosphate/liter. This was prepared by dissolving 128.7

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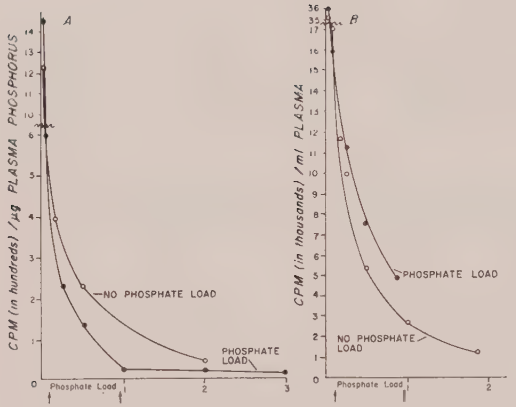

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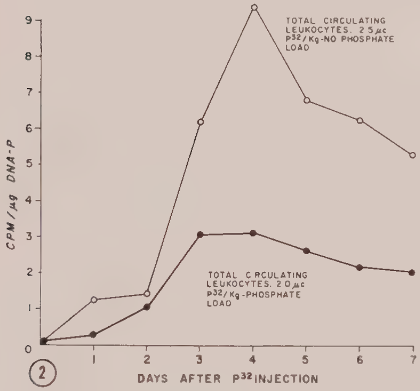

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FIGS. 1, A and B. The effect of a phosphate load on plasma clearance of inorganic P^{32}.

Each curve represents determinations in a single animal.

FIG. 2. The effect of a phosphate load on leukocyte labelling with inorganic P^{32}.

Each point represents a mean value from 4–12 animals.

g of $Na_2HPO_4 \cdot 7H_2O$ and 16 g of $NaH_2PO_4 \cdot H_2O$ in enough distilled water to make 6 liters of solution. The phosphate load, 20 ml of the above solution/kg of body weight in every case, was given intravenously either by drip through a polyethylene catheter or by separate injections of 10 ml aliquots. Loading was initiated between 5 and 10 minutes following P^{32} administration and was completed within 1 hour after beginning. Volumes in excess of 20 ml/kg given within 1 hour frequently produced severe pulmonary edema and could not be used.

Results. The effect of a phosphate load on plasma clearance of inorganic P^{32} following an intravenous dose of 2 μc/kg is shown in Fig. 1-A and 1-B. It is apparent from Fig. 1-A that the phosphate load produced immediate dilution of the specific activity of plasma phosphate. Fig. 1-B shows, however, that the actual rate of clearance of radioactivity from the plasma was decreased by the load. Table 1 compares the specific activities of plasma and leukocyte acid soluble phosphorus from phosphate loaded animals with those from controls. The values from phosphate loaded animals were lower in every case.

DNA-P specific activity of circulating leukocytes from animals loaded with inorganic phosphate after 2.0 μc of P^{32}/kg is plotted in Fig. 2 which presents also for comparison the specific activity curve of DNA-P of circulating leukocytes from animals that received no phosphate load after 2.5 μc of P^{32}/kg. The absolute level of DNA-P specific activity obtained in the loaded animals was less than that produced by the same dose of isotope in animals not given a phosphate load(13) and as Fig. 2 shows no sharp peak in DNA-P specific activity occurred in the loaded animals.

Discussion. This study has shown that a load of non-isotopic phosphate given promptly after injection of P^{32} produces immediate dilution of the activity of the plasma phosphate, decreases the rate of loss of activity from the plasma during the period of loading, diminishes the level of tagging of circulating leukocyte acid soluble and DNA phosphorus, and prevents the sharp peak in DNA-P activity observed in unloaded animals.

It has been shown(13) that with a dose of 0.1 μc of P^{32}/kg no significant activity was present in plasma phosphorus 4 hours after injection and maximum specific activity at 1 minute was only 35.4 cpm/μg of plasma phosphorus. This small dose produced very low labelling of DNA-P (0.8 cpm/μg) in leukocytes from circulating blood on the fourth day after P^{32} administration. It was anticipated that a load of non-isotopic phosphate might quickly dilute the high level of plasma phosphate activity produced by 2.0 μc of P^{32}/kg to the low level induced by 0.1 μc/kg and thereby decrease DNA tagging time. It is apparent that an acutely administered phosphate load does effect a dilution of this magnitude within an hour; however, after cessation

TABLE I. Specific Activity* of Plasma and Leukocyte Acid Soluble Phosphorus after 2.0 μc P^{32}/kg.

Phosphate load		Controls		
Plasma P	Leukocyte acid Soluble P	Plasma P	Leukocyte acid Soluble P	Days after P^{32}
22		42	—	1/12
3.6	1.5	5.4	2.4	1
2.5	2.8	—	4.2	2
—	4.0	—	4.5	3
—	4.8	2.6	5.0	4

* Counts/min./μg of phosphorus. Each value represents a mean from at least 4 animals.

of loading the fall in plasma phosphate activity is quite gradual. The ratio of plasma phosphate activity 24 hours post-injection to that 2 hours post-injection is 0.13 in unloaded animals and 0.16 in those receiving a phosphate load, indicating that rate of plasma clearance after 2 hours is roughly equivalent in both groups. A significant level of plasma phosphate activity persists for at least 2 days (Table 1) in loaded animals. Cellular uptake of phosphate of significant activity can therefore continue and significant labelling of DNA can occur for at least 2 days in animals receiving a phosphate load. As plasma clearance at this time is dilatory, 2 days probably represents a lower limit.

The lower level of tagging of leukocyte acid soluble and DNA phosphorus found in the phosphate loaded animals is not surprising since the label in these fractions comes ultimately from the plasma phosphate. The specific activity of the phosphate of these fractions will be in all cases the resultant of rate of cellular uptake or exchange of phosphate and specific activity of the precursor. As the phosphate load rapidly dilutes the activity of the plasma phosphate a specific activity of cellular acid soluble and DNA phosphorus lower than that found in controls would be expected in the absence of an accelerated rate of uptake in loaded animals during the brief interval when activity of the plasma phosphorus is still relatively high. This seems to be the case.

Fig. 2 shows that administration of a phosphate load immediately after P^{32} injection prevents development of the peak in DNA-P specific activity seen in control animals. The events producing a peak in circulating leukocyte DNA-P specific activity 4 days after injection of label are not clear. In a chemical sense the peak is merely the time when the quantity of labelled DNA-P relative to total DNA-P is maximal. Further, this should be the time when there is circulating the maximum number of cells bearing the highest average level of DNA label per cell. The precise mechanics producing this situation depend upon the availability of labelled precursor phosphate, the kinetics of DNA synthesis, and the rate and manner cells enter into and exit from the circulating blood. There is no evidence that the phosphate load alters either of the latter two, but there is direct evidence that it does affect the availability of labelled precursor phosphate. Therefore, that after 2 hours, rate of plasma clearance in loaded and unloaded animals is roughly the same, suggests that the initial effect of the phosphate load during the first 2 hours after P^{32} injection prevents development of a peak in DNA-P specific activity of circulating leukocytes. This in turn implies that in control animals, the labelling of marrow leukocyte progenitors during the first 2 hours post-injection conditions the occurrence of the peak in circulating leukocyte DNA-P specific activity.

Summary. The effect of an intravenous load of non-isotopic inorganic phosphate following P^{32} on plasma clearance and on leukocyte labelling has been studied. Such a load produces immediate dilution of plasma phosphate activity, decreases rate of clearance of plasma radioactivity during the period of loading, diminishes the level of labelling of circulating leukocyte acid soluble and DNA phosphorus, and prevents development of a peak in DNA-P specific activity. The findings suggest that marrow labelling during the

first 2 hours after P^{32} injection is responsible for development of the peak in DNA-P specific activity found in peripheral blood leukocytes.

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Pyelonephritis. III. Observations on the Association between Chronic Pyelonephritis and Hypertension in the Rat.* (26976)

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The relationship between hypertension and pyelonephritis has not been clearly defined. Various clinical studies have suggested: 1) pyelonephritis can cause hypertension; 2) pyelonephritis can aggravate a pre-existing hypertension and result in "malignant hypertension"; and 3) there is no relationship between renal infection and elevated blood pressure. Experimental consideration of this problem has been somewhat limited by the lack of suitable laboratory model of pyelonephritis. Studies performed in animals with "obstructive" pyelonephritis have with one exception(1) failed to reveal sustained hypertension(3). Similar findings have been recorded in experiments when renal infection was produced by various other manipulative procedures(2,4).

With the availability of a chronic, progressive pyelonephritis in the rat, produced without ureteral obstruction, experiments were designed to study the possible etiologic relationship between kidney infection and hypertension.

Methods. White, male Wistar rats were used. Previous studies have shown that following intravenous injection of a standard inoculum of *Str. faecalis* pyelonephritis uniformly resulted(5). This infection was characterized by the persistence of bacteria in relatively constant numbers in the kidney during the period when the disease progressed from the acute to the chronic stage histologically. In other experiments, microorganisms were found in the majority of rat kidneys

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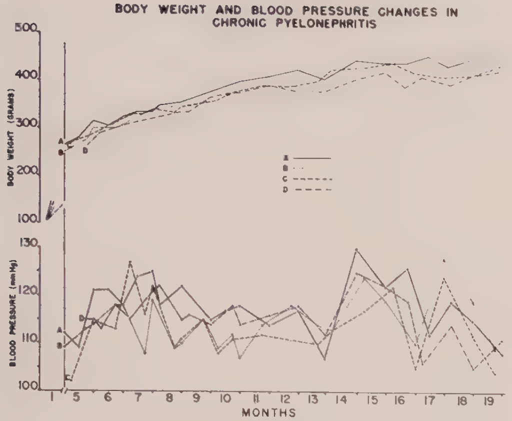

FIG. 1. Body weight and blood pressure recorded in rats with chronic pyelonephritis. Group A—Chronic enterococcal pyelonephritis. Group B—Chronic enterococcal pyelonephritis with acute exacerbations of enterococcal infection. Group C—Chronic enterococcal pyelonephritis with acute exacerbations of *E. coli* infection. Group D—Uninfected controls.

examined 2 years after injection(6).

Four groups of rats were studied in the present experiments. *Group A-Chronic enterococcal pyelonephritis:* Animals received one injection of *Str. faecalis* intravenously. *Group B-Chronic enterococcal pyelonephritis with acute exacerbations of enterococcal infection:* Animals received one injection of *Str. faecalis* intravenously and after an interval of 6 months were given 4 repeated injections of *Str. faecalis* at 2-month intervals. *Group C-Chronic enterococcal pyelonephritis with acute exacerbations of E. coli infection:* Animals were treated as Group B with the exception that the repeated infections were produced by injection of a strain of *E. coli*. *Group D-Controls:* Uninfected animals.

Animals were observed for 20 months. During this time, blood pressures were recorded monthly under light ether anesthesia by the tail plethysmograph method of Williams *et al.*(7). At conclusion of the experiment, rats were sacrificed and weighed. Organs were removed aseptically and prepared for bacteriological or histological study by previously described methods(5).

In the original design of the experiment, 50 animals were included in Group A, 25 each in Groups B, C and D. At the end of the study period there were 33 rats remaining in Group A, 16 in Group B, 21 in Group C, and 16 in Group D. This high mortality noted was not attributed to the renal infection produced or its sequelae, since other studies have shown that rats tolerate this infection well for periods up to 2 years. The majority of deaths in this study occurred in the 24 hours following blood pressure recordings and were therefore attributed to the anesthesia or complications arising therefrom.

Results. Fig. 1 shows the blood pressure and body weight data recorded in these experiments. The animals tolerated the infection well, as noted by normal weight gain. No difference in blood pressure was noted between any of the groups. This failure to measure hypertension was confirmed at autopsy by the finding of normal body weight/heart weight ratios.

The bacteriological data obtained at autopsy are presented in Table I. These results are consistent with those previously published and confirm the persistence of renal infection and the tendency for bacteria gradually to disappear from the liver and spleen following the injection of *Str. faecalis*. The comparatively larger numbers of microorganisms isolated from the organs of Group B animals may be explained by the accumulative effects of the repeated injections of *Str. faecalis* which these rats received. The majority of kidneys examined in Groups A, B and C had macroscopic scars on the cortical surfaces. Histologically these consisted of areas of loss of renal parenchyma, presence of fibrous tissue, mononuclear cell infiltration, dilated tubules filled with "colloid" casts, and

TABLE I. Bacteriological Data Obtained at Autopsy.

Group	No. animals studied	Avg. Log. No. bacteria (per g tissue) kidney	spleen	liver
A	33	3.7	.7	.8
B	16	5.6	1.1	1.2
C	21	2.9* 2.1**	.2	.4
D	16	0	0	0

(*) and (**) represent the average Log. No. of *Str. faecalis* and *E. coli* colonies cultured respectively. Only *Str. faecalis* was isolated from liver and spleen of these animals. Total and differential bacterial counts in this group were obtained by using various inhibitory media; quantitative dilutions were incorporated into agar plates containing Polymyxin, 10 μg/ml, or Vancomycin, 10 μg/ml.

occasionally, minimal to moderate periglomerular fibrosis. Limited alterations were noted in the arteries and arterioles. For the most part these latter were sclerotic changes associated with aging processes and were similar in all groups studied. In a few scarred areas, perivascular fibrosis and mononuclear cells within the vessel wall were noted. These infrequent findings were thought to represent direct extension of the parenchymal inflammatory process to the wall of the arteriole.

Discussion. These results are consistent with most reports concerning the relationship between experimental pyelonephritis and hypertension(2,3,4). Exception to this was noted by Spitznagel and Schroeder(1) who produced pyelonephritis in the rat by intravenously injecting *E. coli* into animals with unilateral ureteral obstruction. Elevated blood pressures were recorded in many of these animals and the authors noted the occurrence of "vascular changes" in the kidneys. This finding may account for discrepancies with other published data. Other investigators have commented upon the absence of significant arteriolar nephrosclerosis and have postulated that this could explain the failure to note hypertension. This was noteworthy in the experiments described here.

Our observations and those recorded in the literature suggest that pyelonephritis does not cause hypertension in experimental animals despite the occurrence of progressive infection, marked tissue destruction and uremia. Whether this represents an animal specie variation is not known. It has suggested an alternative hypothesis to explain the clinically reported increased association between pyelonephritis and hypertension; that hypertension *per se* predisposes to renal infection. Experimental confirmation of this in rats has been reported(8).

Summary. Chronic, progressive, non-obstructed, enterococcal pyelonephritis in the rat is not associated with hypertension. It is suggested that this may be related to the absence of significant arteriolar nephrosclerosis.

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An Antigen for Detection of Hypersensitivity to *Cryptococcus neoformans*. (26977)

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Cryptococcus neoformans exists as a saprophyte in soils of various areas(1,2). The fungus has also been isolated from many specimens of pigeon droppings and nests(3-5), although the organism was not cultured from the internal organs of 20 pigeons(3). The possibility arises, therefore, that a reservoir for human infection can exist in pigeon excreta, for "pigeon" strains and "human" strains possess similar properties of virulence in mice(5).

Human cryptococcosis is world-wide in distribution(6). Most proven cases exhibit a severe meningitis, although pulmonary infection has been rarely found. It has been postulated that for every proven case of coccidioidomycosis there are approximately 2,000 undetected persons harboring the fungus. Perhaps cryptococcosis resembles coccidioidomycosis in this respect. If so, in New York City alone 5,000-15,000 persons could be infected with *C. neoformans*. As a means of proving or disproving this possibility, an epidemiologic survey with an effective skin-testing antigen would be effective(7-9).

Cryptococcal antigens thus far, however, have not been reported to be active in detecting skin hypersensitivity. Antigens, primarily polysaccharide in nature, have been isolated, but have been used essentially in precipitation-type serologic tests(10-12). The development of an effective skin-testing antigen may provide information on prior sensitization with *C. neoformans* and accordingly on the prevalence of undetected cryptococcosis. The present paper describes a method of extracting an antigen which elicits delayed reactions in guinea pigs previously infected with *C. neoformans*.

Materials and methods. Fungus. Two isolates (BET and MAG) of *C. neoformans* were used which originally had been obtained from patients with cryptococcosis. The fungus was maintained at room temperature on Sabouraud's glucose agar. For inoculation into animals or for preparation as antigen, the yeast cells were grown in an amino acid medium at 37°C for 24-48 hours(13).

Animals. White or albino guinea pigs of 300 to 500 g in weight were used. For induction of hypersensitivity, the animals were injected intraperitoneally approximately a week apart with 2 doses each of 5×10^5 living cells of *C. neoformans* in broth. Three weeks to 2 months later, guinea pigs were tested intradermally on the sides or back with 0.1 ml of antigenic solution, and diameters of areas of induration measured 4 hours and 18 to 24 hours after injection.

Method of preparation of antigen. The cells of *C. neoformans* were separated from the growth medium by centrifugation at 3,000 RPM for 1 hour. After having been washed in distilled water until the supernate was clear, the live cells were forced through a tiny aperture of a pressure cell at 60,000 lb/sq. in. (14,15). Subsequent washing and centrifugation served to eliminate much of the capsular material, according to analysis of the sediment under the electron microscope. Pressing the fungus cell suspension through the fine aperture for a second time at a pressure of 60,000 lb/sq. in. caused extensive disruption. The cell mixtures were washed again with several changes of distilled water, and finally centrifuged at 30,000 RPM for half an hour. The resulting sediment was examined under the electron microscope and consisted essentially of fragments of cell walls. This sediment was then resuspended in 50 ml of 0.1 M glycine buffer, pH 10.0, and held with occasional stirring for 2 days at 4°C. The suspension was centrifuged at 3,000 RPM for half an hour, the sediment resuspended in buffer, stirred, and immediately recentrifuged. The combined supernates were neutralized with 1 N HCl, dialyzed against physiologic saline, and filtered through ultra-fine sintered glass. There was no detectable loss of activity on filtration.

Analytical methods. The biuret test(16) was preferred for protein analysis because of its specificity for peptide bonds. When the amount of protein was near the lower limits of significance, analysis with the bromsulphalein method (BSP) was added(17). Egg albumin was used as the reference standard for both tests. Carbohydrate was determined by the modified Molisch test(18), and referred to glucose as a standard.

Results. Analysis. Analysis of typical lots of antigen yielded the following information:

	MAG I	BET IV	BET V
Biuret Protein (as egg albumin)	.06 mg/ml	.11	.38 (.64% of weight of crushed, washed cells)
BSP protein (as egg albumin)	.009 "	.030	.052
Kjeldahl nitrogen	.10 "	.05	.09
Carbohydrate (as glucose)	.090 "	.177	.247
Mean dilution yielding 10 × 10 skin reaction	1/6	1/31	1/96

TABLE I. Areas of Induration in Guinea Pigs Infected with *C. neoformans* and Subsequently Skin-Tested with Soluble Antigen.

Infected with	Diameters at 24 hr after intradermal injection of .1 ml of			
	10 μg/ml	1.0 μg/ml	.1 μg/ml	.01 μg/ml
BET	17 × 18	14 × 14	7 × 7	—
	17 × 17	14 × 13	13 × 12	—
	18 × 18	18 × 18	14 × 12	—
	16 × 17	14 × 17	9 × 11	4 × 4
	24 × 23	13 × 12	13 × 11	7 × 7
	19 × 17	14 × 13	9 × 11	5 × 6
	14 × 14	10 × 12	11 × 10	0
		11 × 15	11 × 10	7 × 9
		11 × 11	11 × 10	3 × 3
MAG	20 × 20	14 × 14	9 × 10	0
	15 × 15	10 × 10	8 × 9	6 × 6
	14 × 18	13 × 13	8 × 9	8 × 9
	18 × 18	12 × 12	8 × 9	6 × 6
	16 × 18	13 × 15	11 × 10	8 × 9
	28 × 28		17 × 17	
	17 × 17		14 × 14	

Intradermal tests. Guinea pigs were tested intradermally with varying dilutions of the soluble supernate; doses were measured according to the amount of protein (biuret) (Table I). Of 3 different lots of antigen from the BET isolate the mean value of dilutions which induced at least 10 × 10 mm induration in infected guinea pigs represented about 0.4 μg of protein (biuret) per animal. Fifteen guinea pigs hypersensitive to *Candida albicans,* 20 hypersensitive to *Histoplasma capsulatum,* and numerous normal ones did not develop reactions to intradermal injection of any of the *C. neoformans* antigen preparations.

A soluble antigen was prepared from a second isolate of *C. neoformans* (MAG).* Complete cross-reactivity occurred between animals infected with one strain and skin-tested with the other.

Two lots of *C. neoformans* were killed with ether (2 parts ether to 1 part broth) or 1:5000 merthiolate without treatment in the pressure cell. Extracts obtained from these with 0.1 M glycine at pH 10 had much higher carbohydrate content, but were low in protein and had no skin-testing activity.

Discussion. A soluble antigen was prepared from *C. neoformans* by crushing the yeast cell, discarding the supernatant and then extracting the insoluble portion. After the first exposure to high pressure, examination of the cells under the electron microscope revealed that most of the capsules had been removed from the cells. After these cells had been further washed, a second exposure to high pressure followed by extraction with 0.1 M glycine at pH 10.0 produced a solution which contained a skin-testing antigen. This technique of disrupting the live cells before extraction yielded an antigen relatively low in capsular polysaccharide. When the cells were killed with ether or merthiolate and then extracted, the carbohydrate content was much higher but skin-testing activity was absent.

The skin-testing antigens, prepared separately from 2 isolates, reacted similarly in animals infected with either homologous or heterologous isolate. Obviously, further experimentation is required before the importance of strain differences can be properly evaluated.

The aforementioned antigenic complex of *C. neoformans* appears to be effective in detecting hypersensitivity in guinea pigs due to prior infection. Further studies are now in progress to ascertain the activity of the antigen in man and the possible presence of benign cryptococcosis in human populations.

Summary. An antigen has been prepared from *Cryptococcus neoformans* which induces delayed skin reactions in guinea pigs previously infected with this fungus. Cross reactions did not occur in animals infected with *Candida albicans* and *Histoplasma capsulatum.*

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Role of Infection in the Delayed Deaths of Mice Following Extensive Burn Injury. (26978)

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This study is concerned with the role of infection in the delayed deaths of mice following thermal burns; it does not deal with the problem of shock encountered during the first 2 days following injury. Extensive studies with experimental animals exposed to irradiation or to traumatic shock indicate that a reduction in natural resistance to infection follows this type of injury(1-5). In patients with extensive burn injury, the initial traumatic shock may be followed later by shock accompanying bacterial invasion and generalized "burn decompensation"(6-9); although the injured tissues may suffer localized infection within the first 4 days, invasion of the deeper tissues seldom occurs before the 5th post-burn day(10,11).

The delayed mortality in mice following extensive burn injury has long been investigated in our laboratories(12,13). This mortality occurs during the first 3 weeks after injury in animals surviving the initial period of traumatic shock. Therapy with 0.9% NaCl solution, given subcutaneously or intraperitoneally in amounts equivalent to 15% of body weight, produces over 85% survival at the end of the 2nd day. The mortality rate rises rapidly during the first 10 days but may not reach its maximum (approximately 75%) until the 3rd week of injury. The toes, tails and skin of the burned area become gangrenous and may slough off. Food consumption decreases and the animals become emaciated. No significant changes have been observed in body temperature, in the circulatory system (hematocrit, bleeding volume and plasma proteins), or in blood NPN levels. None of the histopathological changes observed in lungs, liver, spleen, adrenals, pancreas and kidneys of burned mice have been adequate to explain the deaths(14). Our studies indicate that infection is an important factor in the cause of delayed deaths of burned mice. Moreover, Rosenthal(14) and Millican and coworkers(15,16) have demonstrated that some chemotherapeutic agents are effective in promoting the survival of these burned mice.

Methods. Female albino mice (NIH or GP strains), weighing 16-22 g were employed. Burn trauma was produced by standardized procedures previously described(12,13). Anesthetized animals were dipped to the axilla in water at 70°C and held there for 8 seconds. This degree of injury is rapidly fatal to over

85% of the mice in 24 to 48 hours if they remain untreated and so 0.9% NaCl solution was given either intraperitoneally or subcutaneously in amounts equivalent to 15% of body weight. They were housed in glass jars in groups of 5 on dried sugar cane bedding.

In 3 experiments a total of 97 mice were available for observations on delayed deaths. To assess the role of infection, cultures were made of the various tissues of each of 43 of these mice selected at random and sacrificed at varying intervals after injury. Nineteen animals in Series 1 were killed on the 4th to 7th post-burn days; 10 in Series 2 were sacrificed on the 3rd and 4th post-burn days; and 14 in Series 3 on the 2nd, 3rd, 5th and 9th post-burn days. In Series 1 and 2 the mice were killed as soon as jar mates began to die but before they became moribund; in Series 3 although more than half of the deaths occurred after the 9th day, 11 of the mice were sacrificed on or before the 5th day. A 4th group of 10 supposedly normal mice was selected at random from the large colony and sacrificed as controls on our techniques of sampling tissues. Since no deaths were occurring among the stock mice and since no bacteria usually associated with the more common endemic infections in such laboratory colonies had been identified in a study of bacteria isolated from 33 or more burned mice just before this investigation was begun, it was not considered essential to culture the organs of a large number of stock animals.

The burned mice, especially those in Series 1 and 2, were usually too sick to move about the jars readily and consequently the denuded, necrotic surfaces of the burned tissues and gangrenous extremities were constantly subjected to contamination by fecal droppings that collected rapidly in the bedding. Their fur was visibly soiled.

Relatively large pieces of tissues were examined. Samples of muscle about 1 g in weight were cut from an area adjacent to an injured surface in each thigh of all burned mice and from a similar area in the control mice. The thoracic cavities were opened under aseptic conditions first and the exposed tissues sampled. Then the peritoneal cavities were entered. The peritoneal fluid was cultured as soon as the cavity was opened. Relatively large samples of the organs were removed and placed directly in broth: the whole heart was divided and half placed in each of 2 tubes, 1 lobe of a lung, no less than half the spleen, a piece of liver equal in size or larger than the sample of spleen, and 1 kidney. Any capsular material surrounding the tissues was severed in several places with sterile scissors.

All materials were cultured in about 10 ml of neopeptone broth in tubes. In addition, the hearts (blood and tissue) of most of the mice and also thigh muscles from each leg of all mice were cultured in deep dextrose brain medium or Robertson's ground meat medium under vaseline seals so that adequate anaerobiosis would be provided for isolation of obligate anaerobes. All cultures were observed for the growth of organisms and each was plated on blood agar as well as on infusion agar after 24 and 48 hours incubation at 37°C. Positive cultures were studied in appropriate media for further identification. Since these studies were made before some of the more refined methods for controlling the overgrowth of spreaders were developed and found so effective, they were not available for use.

Results. Bacteria were isolated from the heart, lungs, liver, spleen and/or kidneys of 40 of the 43 burned mice; 3 of the mice of Series 3, which reacted less severely to the burn than those in the other 2 series, were apparently more resistant to penetration of bacteria from infected foci. Twenty-eight of the 40 had positive heart cultures. These and other findings are shown in Table I. It is probable that the tissues which harbored several types of bacteria were actually infected with only one or 2 strains; the others were no doubt present in extremely small numbers, and not playing a major role in the infection. The striking observation was the finding of bacteria in most of the tissues of each injured mouse; the heart cultures, with 70% positive, contained organisms less often than those of the other tissues, and the lung cultures, with 88% positive, were the ones that most frequently showed growth. This high incidence of bacteria in the lung tissues may have been due to the presence of latent infection in the lungs of these animals at time of burn injury.

It appears that bacteria found ample opportunity for colonization on the damaged sur-

TABLE I. Incidence of Bacteria in Cultures of Tissues of Burned Mice and of Mice Used as Controls on Sampling Techniques.

Mice	Tissues cultured	Results of culturing							
		Cultures not made	Negative cultures	Positive cultures					
				Total	1 Genus	2 Genera	3 Genera	4 Genera	5 Genera
43 Burned	Heart	0	15	28	19 (7)[1]	9	0	0	0
	Lung	2	5	36	25 (8)[1]	8	2	1	0
	Liver	1	9	33	22 (12)[1]	8	3	0	0
	Spleen	2	12	29	22 (12)[1]	6	1	0	0
	Kidney	2	11	30	23 (13)[1]	5	2	0	0
	Combination of Organs from ea. Mouse	0	3	40	15 (8)[1]	13	6	4	2
10 Controls on Sampling Tech.	Heart	1	9	0	0	0	0	0	0
	Lung	1	8	1	1[2]	0	0	0	0
	Liver	1	9	0	0	0	0	0	0
	Spleen	0	8	2	2[3]	0	0	0	0
	Kidney	0	7	3	3[4]	0	0	0	0
	Combination of Organs from ea. Mouse	0	4	6	6	0	0	0	0

1. Numbers in parenthesis indicate number of cultures included in each group which contained spreaders and which consequently may have contained additional species that were not detected in subcultures.
2. Aerobic Gram-positive spore-forming rod.
3. *Proteus* (1), *E. Coli* (1).
4. Anaerobic Gram-positive rods (2), diphtheroid (1).

faces, were successful in extending their growth to adjacent tissues and eventually accomplished generalized invasion. Bacteria were isolated from cultures of thigh muscles of 44 of 47 burned mice; the negative cultures were from mice in Series 3. In general, the species isolated from the thigh muscles were the same as those predominating in the cultures of heart, lungs, liver, spleen and kidneys of 30 of 35 burned mice for which data were comparable.

In studies of this type the questions of extensive terminal invasion from the respiratory and gastro-intestinal tracts of moribund animals, and of gross contamination during handling of specimens must be considered. Since every effort was made to select mice that were not moribund and since approximately 75% of the cultures of peritoneal fluid were sterile, it has been assumed that in most instances the isolations reflect a reduction in the natural resistance to invasion of organisms multiplying in burned surfaces or from latent foci in deeper tissues. While the cultures of muscle tissue from control animals indicate that some contamination did occur during the taking of the samples, it was limited. It appears that the possibility of skin contaminants may enter into any interpretation of the results of cultures of muscle tissue, but is of much less significance in evaluation of cultures of internal organs.

The organisms most frequently isolated from the internal organs of the burned mice belong to the following genera: *Staphylococcus, Streptococcus, Pasteurella, Proteus* and *Escherichia*. Their frequency of occurrence varied from 15 to 24% of the isolations. Diphtheroids, *Aerobacter cloacae* and *Pseudomonas aeruginosa* were demonstrable in less than 10% of the isolations.

It is of interest that strains of *Pasteurella* were isolated from 16 of the 43 mice; they were isolated from heart blood 11 times, from the lungs 9 times and from the kidneys twice. Overgrowth by other organisms probably interfered with more frequent isolation. These strains had the morphological and biochemical characteristics of the pneumotropic strains which were isolated from several colonies of mice, including some from the Nat. Inst. Health, and described by Jawetz(17). This organism was found to exist as a latent infection in the respiratory tract of mice and appeared to be widespread in mouse colonies (18).

All of the streptococci appeared to belong to the enterococcus group; of the 15 isolations, 10 were *alpha* hemolytic and 5 were *beta* hemolytic strains. While no serological grouping was done, the biochemical characteristics of all strains were sufficiently similar to those of *Streptococcus faecalis liquefaciens* or *Streptococcus zymogenes* for them to be tentatively classified with these species.

Discussion. It would appear from the results of the experimental work that generalized bacterial invasion of the tissues may occur during the first 10 days following extensive burn injury in mice. Systemic infection must have been the cause of the delayed deaths of many of the mice. The physiological state of the host and the biochemical nature of the burned surfaces were, no doubt, as important determinants(19) in the spread of infection as the virulence of the organisms or any reduction in the effectiveness of the natural cellular and humoral defense mechanisms. Non-specific factors such as nutritional inadequacies(20) and environmental humidity(21) probably played a role. While the predominating organisms demonstrable in the tissues were ones ordinarily considered of low invasiveness and virulence, it is well established that bacteria undergo rapid and profound changes in cultural characteristics and pathogenic properties as a result of physicochemical factors in their environment. Goshi, Cluff, Johnson and Conti(22) have found that induction of infection of the burned skin of rabbits within 3 days after injury required only 1/10 the dose of coagulase-positive staphylococci necessary to produce infection in normal rabbit skin. Blood cultures and autopsy examination of these animals did not show bacteremia or metastatic infection, but the burned area in the rabbits was minimal in comparison with that in the injured mice in our series; the effect of the burn was localized to the site of injury and the host defenses continued to operate. They(23) also found that in rabbits immunity to infection by pathogenic staphylococci in necrotic burns could be induced by vaccination and this immunity was passively transferable with rabbit antiserum. These findings are in agreement with those of Rosenthal and Millican (14,15,16) who have reported that chloramphenicol therapy is partially effective in protecting against delayed deaths, and that human gamma globulin as well as the serum of animals convalescing from burn injury possess protective activity against deaths(14,15). The data add additional support for the role of infection in the cause of deaths in the burned mice.

There is some question as to whether the mouse may be more susceptible to the establishment of generalized infection following thermal injury than are other animals, due to the less effective bacteriacidal properties of the serum toward both Gram-positive and Gram-negative organisms(24,25). Muschel and Muto(26) as well as Kornfeld, Hammond and Miller(27) have shown that bactericidal antibody, such as that found in other animals,. is present in the normal mouse and lack of bactericidal action may be attributed to a lack of complementary activity(26,28). The incidence of bacteremia is not such a prominent phenomenon in dogs, rabbits and guinea-pigs following irradiation as it is in mice(29,30). Reports suggest that while sublethal radiation has been found to activate subclinical or latent infections in mice, the findings are not as definite in rats. However, in humans with extensive burns, there is a rising incidence of deaths from septicemia due to *Pseudomonas aeruginosa* or to *Staphylococcus aureus*, (7,8,9) and frequently visceral lesions are present(31).

Summary. Studies have been described in which generalized infection appeared to be an important factor in the delayed deaths in mice during the 3 weeks following extensive burn injury. Bacteria of low virulence and invasiveness apparently spread both from previously localized foci of latent infection and from areas of colonization on damaged surfaces.

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Correlation between Heat-Resistance of Polioviruses and Other Genetic Markers.* (26979)

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The study of the genetic markers of poliovirus has assumed added importance as a result of the wide use of attenuated viruses as vaccine. The association of certain tissue culture markers with the presence or absence of neurovirulence and the stability of these traits has been investigated intensively. The present study deals with the resistance of polioviruses to heating at 50°C and correlation of this property with 2 other widely used markers, efficiency of plating at low bicarbonate concentrations(1) and capacity to multiply at 40°C(2).

Materials and methods. Viruses. Sixteen strains representing the 3 immunologic types of poliovirus were studied. Three strains (SI, SII, SIII) were provided by Dr. Joel Warren and 10 strains were obtained through the courtesy of Dr. J. L. Melnick (PII, PIII, MIA, MIB, MIC, MID, MIIIA, MIIIB, MIIIC, MIIID). The other 3 strains (Mah, MEF-1, Sau) were carried in this laboratory.

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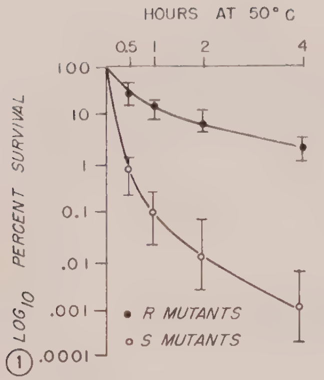

FIG. 1. Inactivation curves at 50°C of S and R mutants of Type 1 poliovirus strains. Range of survival and mean values for six strains.

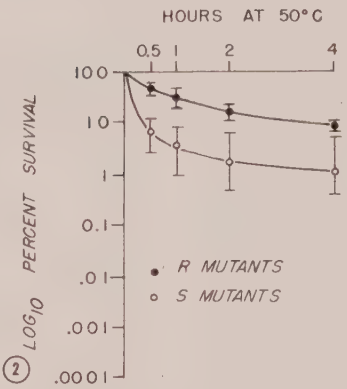

FIG. 2. Inactivation curves at 50°C of S and R mutants of Type 2 poliovirus strains. Range of survival and mean values for three strains.

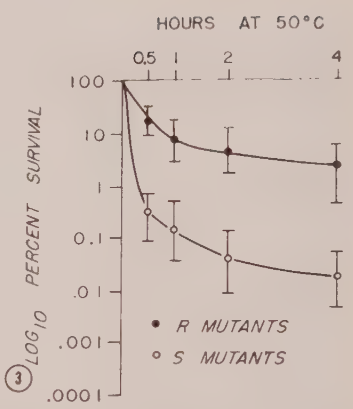

FIG. 3. Inactivation curves at 50°C of S and R mutants of Type 3 poliovirus strains. Range of survival and mean values for seven strains.

Virus cultivation and plaque assays were done in primary monkey kidney cultures as previously described(3,4). Single plaque progeny lines of all virus strains were used in these experiments.

Thermal inactivation of virus at 50°C was carried out as previously described(5). Thermal resistant (R) mutants were selected from sensitive (S) parent virus by 4 cycles of alternate heating at 50°C for 8 hours and propagation of the survivors in monkey kidney cell cultures.

Tissue culture markers were studied by methods which have been previously reported. The *T marker,* capacity to grow at 40°C, was determined by the method of Lwoff and Lwoff(2). The *d marker,* efficiency of plating at low bicarbonate concentration, was measured by the technique of Hsiung and Melnick(6). As control, known d^+T^+ and d^-T^- strains were included in all tests.

Results. The rates of inactivation at 50°C of parent viruses and corresponding R mutants of 16 strains representing the 3 immunologic types of poliovirus were studied. Comparison of the inactivation curves (Fig. 1,2,3) of the parent viruses shows that distinct differences can be found in rates of inactivation of the 3 immunologic types. Type 1 strains are most sensitive to 50°C inactivation, type 2 strains are least sensitive, and type 3 strains occupy an intermediate position. These results confirm data obtained previously with 16 other strains(5). It was possible to select 50°C-resistant (R) mutants from all parent viruses. The parent viruses, therefore, are referred to as sensitive (S) mutants. The data illustrated in Fig. 1, 2, and 3 show that the inactivation curves of the R mutants are similar for all 3 types of poliovirus.

The results in Table I illustrate 2 important points. First, parent (S) viruses which have different d and T markers show inactivation curves characteristic of their immunologic type. For example, of the 6 type 1 parent viruses studied, 3 were d^+T^+ and 3 were d^-T^-. It can be seen from Fig. 1 that all 6 showed essentially the same inactivation characteristics when heated at 50°C. This is also true of the type 2 and type 3 strains studied. Second, Table I shows that when R mutants from all 16 strains were selected, the d and T markers did not change.

Discussion. The susceptibility to inactivation of polioviruses at 50°C has been designated by Dulbecco(7) as either t^s (sensitive mutant) or t^r (resistant mutant). It was suggested(7) that there is an extensive degree of covariation of this marker with the d and T, as well as some other markers. The data presented here indicate that there is no correlation between rate of inactivation at 50°C of the 16 strains studied and their d and T markers. The susceptibility to inacti-

TABLE I. Comparison of d and T markers of S and R mutants of 16 strains of poliovirus.

	Type 1														Type 2						Type 3																
Strain	Mah		SI		MIA		MIB		MIC		MID		MEFI		SII		PII		Sau		SIII		PIII		MIIIA		MIIIB		MIIIC		MIIID	
Mutant (50°C)	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
d marker	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	±	±	−	−	+	+	−	−	−	−	−	−	−	−
T "	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	±	±	+	+	−	−	−	−	−	−	+	+

vation at 50°C of polioviruses is more related to their immunologic type than to the other markers. For example, the inactivation rate of type 2 viruses is significantly slower than the inactivation rates obtained with type 1 and type 3 viruses. Furthermore, the resistant mutants showed the same d and T markers as the sensitive parent viruses from which they were selected. These results clearly support the conclusion that resistance to inactivation at 50°C varies independently from the d and T markers.

It is interesting to note that all 16 parent virus strains studied were clearly t^s in regard to their inactivation at 50°C while differing in their d and T characters. From all strains, t^r mutants could be selected which did not differ significantly in their inactivation rates. Since the selection of these t^r mutants did not alter the other characteristics measured, it is suggested that t^r mutants could be used as an additional criterion for identifying and studying the stability of attenuated poliovirus strains. It would also be of interest to determine whether there is any correlation between the degree of neurovirulence and the $t^s \rightarrow t^r$ mutation.

Summary. The genetic marker involving resistance of polioviruses to heating at 50°C is independent of the capacity of mutants to multiply at 40°C or their plating efficiency at low bicarbonate concentrations. The possible usefulness of heat-resistant mutants for the study of attenuated poliovirus strains is discussed.

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The Effect of Trypsin on Measles Virus Antigenicity. (26980)

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Recent studies in this laboratory have been directed toward the concentration and purification of measles virus. In the purification studies, enzymatic digestion, which has been used in a number of attempts to purify other viruses(1-5), was investigated. Trypsin, in particular, has been used effectively(2-4). Gresser and Enders(6) have noted the destruction of infectivity and the hemagglutinins of some myxoviruses by trypsin. The experiments reported here demonstrated the deleterious effects of trypsin on the capacity of measles preparations to evoke neutralizing and complement fixing antibodies in guinea pigs.

Materials and methods. Virus. The Philadelphia 26 strain of measles virus, isolated in this laboratory(7), was used for preparation of measles virus-infected fluids in grivet monkey kidney cells. This strain was also passed in stable human amnion cells (8) and used as challenge virus in the serum neutralization test.

Tissue cultures. Trypsinized primary monkey kidney epithelial cultures were grown in medium 199 with 2% calf serum. Bottles were re-fed with the same medium on the fifth or sixth day and infected with measles virus on the seventh day. Following infection, cell cultures were maintained in Eagle's basal medium prepared with Hanks' salt solution (HBME) without serum.

TCF. Fluid was drawn off from settled cell debris on seventh day post inoculation.

Whole TCF. Fluid plus cell debris, seventh day post inoculation.

Serum neutralization test. Stable human amnion cell cultures(8) were used for serum neutralization tests. The cells were dispensed in 1 ml amounts, 35,000 cells per ml, in a medium consisting of Eagle's basal medium prepared with Earle's salt solution (EBME) with 5% calf serum and tubes incubated in stationary racks. The following day the medium was removed and replaced with 2 ml of fresh medium. Serial dilutions of inactivated serum (56°C—30 minutes) in medium 199 at pH 6.8 were incubated for one hour at room temperature with an equal volume of measles virus suspension containing 100 $TCID_{50}$ per 0.1 ml. Following incubation, 0.2 ml of each mixture was added to each of 2 cell culture tubes. The cultures were incubated for 7 days at 36-37°C at which time they were observed for measles cytopathic changes. The titer was calculated as the highest initial dilution of serum which caused complete or near complete suppression of cytopathic change.

Complement fixation (CF) test. The complement fixation (CF) tests were performed according to Osler *et al.*(9), employing five 50% hemolytic doses of guinea pig complement and overnight fixation in the cold. For titration of antigen, four 50% (H_{50}) hemolytic units of guinea pig immune serum were employed. A suspension of partially purified and concentrated measles virus antigen was incubated in each test as a control. CF units, as used in this paper, are defined as the reciprocal of the dilution of antigen required to yield 50% fixation in this test procedure.

Sedimentable CF antigen. CF antigen which is pelleted by one hour centrifugation at 105,000 × g (40,000 rpm, no. 40 rotor, Model L centrifuge (Spinco)).

Non-sedimentable CF antigen. CF antigen remaining in the supernatant fluid after one hour centrifugation at 105,000 × g.

Nitrogen determination. Nitrogen was determined as described by Johnson(10).

Immunogenic potency test. Guinea pigs weighing 400-500 g were used for immunogenic potency tests. Immunization schedule consisted of three 1 ml intramuscular injections of each preparation given at weekly in-

TABLE I. Release of Measles CF Antigen* from Infected Cell Debris.

Description of sample	CF units of clarified fluid	CF units of 105,000 x g supernate	CF units 105,000 x g pellet at equal vol.	% Additional CF antigen (sedimentable)
Clarified TCF	9	<1.7	5.6	—
Suspended cells incubated 1 hr at 37°	<.5	<.2	<.4	<7
Frozen and thawed cells incubated at room temp. 1 hr	<.5	<.2	<.4	<7
Frozen and thawed cells incubated 1 hr at 37°	<.5	<.2	<.4	<7
Frozen and thawed cells 200 μg/ml trypsin, incubated 1 hr at 37°	**	**	4.4	79

* CF values calculated back to original TCF vol.
** Anticomplementary.

tervals. Bleedings were taken 7 days after the third injection.

Results. Release of additional CF antigen. An attempt was made to obtain additional CF antigen from the cell debris at the usual harvest time (seventh day post-seeding). Five hundred ml of whole TCF was centrifuged at low speed and the supernatant TCF decanted. The packed cell debris was washed with 0.15 M NaCl at 5°, and the wash removed by low speed centrifugation. The washed cell debris was suspended in 160 ml of 0.15 M NaCl. A one-fourth volume aliquot of this suspension was set aside without further treatment. The remainder was frozen at —20° and thawed at 37° for a total of 3 cycles. This frozen-thawed suspension was divided into 3 equal parts. One was kept at room temperature for one hour, the second was held at 37° for one hour (as was the original cell suspension), and the third was incubated at 37° for one hour with 200 μg/ml of crystalline trypsin (Worthington). At the end of the one hour period, all 4 aliquots were clarified by low speed centrifugation. A portion of each clarified aliquot was then subjected to one hour centrifugation at 105,000 × g (40,000 rpm, no. 40 rotor, Model L centrifuge (Spinco)). The supernatant fluids were decanted and the pellets suspended in 0.063 M potassium phosphate buffer, pH 7.0, containing 2% NaCl. The results of a representative experiment of this type are shown in Table I.

The data in Table I indicated that an additional 79% (calculated on the basis of the CF antigen sedimented from the clarified TCF) of sedimentable CF antigen was obtained from the cell debris by digestion with trypsin. This CF antigen had not been released into the TCF during the virus growth cycle and was not released from the cell debris by 3 cycles of freezing and thawing. A second digestion with trypsin did not release additional CF antigen. Later work indicated that as little as 5 μg of trypsin per ml of whole TCF was sufficient to release CF antigen. Release of additional CF antigen was similar in magnitude whether or not the cell debris had been frozen and thawed prior to digestion with trypsin. For convenience, subsequent digestions were conducted using whole TCF, rather than separated cell debris, without prior freezing and thawing. Tryptic digestion of infected tissue culture fluid free of cell debris resulted in no change in CF activity. A summary of the results of tryptic digestion of whole measles TCF (10 lots) is shown in Table II. An average increase in measles CF antigen of 138% was observed. All the CF antigen was sedimentable (105,000 × g for one hour). No non-sedimentable antigen was ever observed under the conditions employed.

Higher digestion temperature. Digestion of whole TCF with trypsin, in addition to releasing additional CF antigen, also aided purification by converting contaminating protein to dialyzable digestion products. From the knowledge of the properties of trypsin, it was expected that an increase in the temperature and duration of digestion would increase

TABLE II. Results of Tryptic Digestion* of Whole TCF.

Lot no.	Original fluid (CF units)	Digested fluid (CF units)	% Increase
13	2.8	5.7	103
14	1.0	3.4	240
17	5.7	13.5	137
18	3.4	9.5	180
21	3.4	8.0	135
22	2.8	5.7	103
26	2.8	6.8	142
Pool of 29 and 34	2.0	5.7	185
“ “ 30, 32, 33, 35	4.8	13.5	182
“ “ 40, 42, 45	5.7	8.0	40

* All digestions were performed at 37°, 1½–2 hrs, 10 μg/ml trypsin.

the proteolytic effect. Experiments were, therefore, conducted in which whole TCF was digested at 45° for various time periods with 10, 30 or 50 μg/ml of trypsin. The results are shown in Table III.

The data in Table III indicated that incubation at 45° for up to 24 hours (this was extended to 48 hours in later experiments) did not destroy the CF antigen or convert it to non-sedimentable CF antigen. The use of trypsin at this elevated temperature produced a considerable increase in CF antigen while eliminating extraneous protein as shown by the increase in CF per μg N values. Since little, if any, difference existed in the range of 10 to 50 μg/ml trypsin, 30 μg per ml was arbitrarily selected for use.

Potency tests. To determine whether the the proteolytic digestion procedure had affected the antigenic potency of the measles virus, samples were prepared for assay in guinea pigs as indicated below:

Sample A—whole TCF clarified by low speed centrifugation
Sample B—whole TCF kept at 45° for 24 hours, then clarified
Sample C—whole TCF incubated at 45° for 24 hours with 30 μg/ml trypsin, then clarified
Sample D—suspension of ultracentrifugal pellet from A
Sample E—suspension of ultracentrifugal pellet from B
Sample F—suspension of ultracentrifugal pellet from C

The ultracentrifugal pellets were obtained by one hour centrifugation at 105,000 × g and resuspended in 0.15 M NaCl to yield essentially the same CF titer as the fluid from which they were derived. The potency test was conducted as described under *"Materials and Methods"*. The results are shown in Table IV. These data indicate the following: 1. The measles immunogen is stable to heat at 45° for 24 hours. 2. The immunogen is sedimentable from heated (45°) or unheated fluids at 105,000 × g for one hour. 3. The immunizing capacity is reduced by digestion with trypsin at 45° for 24 hours, as measured in guinea pigs. 4. CF antigen (sedimentable) is not necessarily antigenic in guinea pigs and, therefore, sedimentable CF antigen is not necessarily a measure of measles immunogen.

Since the loss of antigenic potency result-

TABLE III. Results of Tryptic Digestion of Whole TCF at 45°.

Trypsin (μg/ml)	Time at* 45° (hr)	Fluid clarified post-incubation (CF units)	Ultracentrifugal supernate (CF units)	Ultracentrifugal pellet (CF units)	Specific activity of pellet (CF units/μg N)
0	0	4.0	<.7	2	.44
0	2	4.8	<.7	4	.93
0	5	5.7	<.7	4	.92
10	2	11.5	<.7	14	4.1
10	5	13.5	<.7	16	8.2
10	24	16.0	<.7	14	12.2
30	2	16.0	<.7	14	5.9
30	5	16.0	<.7	14	5.7
50	2	13.5	<.7	12	4.8
50	5	16	<.7	12	5.5
50	24	19	<.7	16	15.2

* Control sample at 24 hr with no trypsin and 24 hr sample at 30 μg were lost through breakage. All values calculated to original TCF vol.

TABLE IV. Effect of Tryptic Digestion at 45° on Immunogenicity of Whole TCF.

Description of sample	Dilutions injected	Serological conversions obtained Neutralization*	 Complement fixation**
A. Whole TCF, clarified	1:5	3/3	3/3
CF units = <3.5	1:25	4/4	3/4
B. Whole TCF kept at 45° for			
24 hr, then clarified	1:5	2/2	2/2
CF units = <3.5	1:25	3/4	3/4
C. Whole TCF kept at 45° for			
24 hr with 30 μg/ml			
trypsin, then clarified	1:5	0/3	0/3
CF units = 5	1:25	0/3	0/3
D. Resuspended ultracentrifugal	1:5	4/4	4/4
pellet from (A)	1:25	3/3	3/3
CF units = 7	1:125	3/3	3/3
E. Resuspended ultracentrifugal	1:5	3/3	3/3
pellet from (B)	1:25	3/3	3/3
CF units = 7	1:125	3/3	2/3***
F. Resuspended ultracentrifugal	1:5	0/4	1/4
pellet from (C)	1:25	0/4	0/4
CF units = 14	1:125	0/2	0/2

* No. of guinea pigs with neutralizing titer of 1:2 or greater/no. on test. Range of titers was from 1:2 to 1:64.

** No. of guinea pigs with CF titer of 1:5 or greater/no. on test. Range of titers was from 1:5 to 1:160.

*** One serum anticomplementary.

ing from the use of 30 μg/ml of trypsin at 45° for 24 hours was so marked, a similar potency test was conducted to evaluate the effect of the less rigorous conditions (10 μg/ml trypsin, 37°, 1-3 hours) which had been used in earlier parts of this study (Table II). The results of this experiment are shown in Table V. The adverse effect of trypsin on the measles antigen(s) is still apparent, even when digestion was conducted for only one hour.

Discussion. The data reported here have shown that digestion of whole measles TCF or the infected cell debris therefrom with trypsin at 37° or 45° results in a 2-3 fold increase in the amount of sedimentable (105,000 × g) CF antigen. Although it might have been expected that such an in-

TABLE V. Effect of Tryptic Digestion at 37° on Immunogenicity of Whole TCF.

Description of sample	Dilutions injected	Serological conversions obtained Neutralization*	 Geometric mean titer***	 Complement fixation**	 Geometric mean titer***
Whole TCF kept at 37° for					
3 hr, then clarified	1:5	8/8	50	7/8	49
CF units = 14	1:25	7/7	21	7/7	30
Whole TCF kept at 37° for					
1 hr with 10 μg/ml					
trypsin, then clarified	1:5	7/7	22	7/7	20
CF units = 38	1:25	3/6	4	3/6	5
Whole TCF kept at 37° for					
3 hr with 10 μg/ml					
trypsin, then clarified	1:5	4/8	2	2/8	2
CF units = 38	1:25	0/8	—	0/8	—

* No. of guinea pigs with neutralizing titer of 1:2 or greater/no. on test. Range of titers was from 1:4 to 1:128.

** No. of guinea pigs with CF titer of 1:5 or greater/no. on test. Range of titers was from 1:5 to 1:320.

*** In calculating geometric mean titers for those groups with at least one conversion, undetectable neutralization titers and CF titers of <5 were arbitrarily assigned a value of 1.

crease would be paralleled by an increase in antigenic potency, tests in guinea pigs showed that the digestion with trypsin at either 37° or 45° resulted in a marked loss of potency. These data also confirmed the stability of the measles immunogen at 45° (without trypsin) reported previously(11).

In relation to the purification and concentration of measles virus, it is clear that the CF property of measles virus preparations should not be used to evaluate such procedures without frequent animal potency tests.

Summary. Digestion of whole measles fluids with crystalline trypsin at either 37° or 45° results in a 2- to 3-fold increase in amount of sedimentable complement fixing antigen. This treatment, however, markedly reduces the ability of these fluids to induce formation of neutralizing or complement fixing antibodies when injected in guinea pigs.

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A Salmonellosis Resistance Factor For the Guinea Pig.* (26981)

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A relationship between nutritional status and resistance to disease has long been recognized but the nature of this relationship remains obscure(1). Schneider(2) observed decreased mortality among mice infected with *Salmonella enteriditis* when a purified basal diet was supplemented with whole wheat, malted barley or dried egg white. Mortality among guinea pigs subjected to whole-body X-radiation was significantly reduced when the animals consumed raw cabbage or broccoli(3), but it is not clear whether or not the increased survival was the result of increased resistance to infection. Preliminary observations made in our guinea pig colony suggested that consumption of raw cabbage decreased mortality due to salmonellosis. To test this effect under controlled conditions guinea pigs fed a purified diet, with and without cabbage supplementation, were artificially infected and the observations are reported here.

Methods. Female guinea pigs were reared from weaning on the purified basal diet described below or on the basal supplemented with cabbage or cabbage fractions. In so far as possible they were continued on the same diets during the experimental period, but in all cases were fed the experimental diets for at least 2 weeks prior to infection.

Two groups of animals, 10-16 weeks of age and weighing 400 to 600 g, were used in each of 5 trials. They were kept in cages with raised wire floors and housed in a room maintained at 75 ± 5°F. All received a basal purified diet and one-half received in addition a daily supplement of cabbage, *ad libitum*. The basal diet contained, in percent, washed casein 30.0, sucrose 43.3, wood pulp 15.0, salts(4) 4.0, soybean oil 4.0, po-

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* Contribution from Missouri Agri. Exp. Station. Approved by the Director. Journal Series No. 2329. Supported in part by Nat. Science Foundation Grant.

TABLE. I. Cabbage supplementation and resistance to salmonellosis

Observations	Basal diet	Cabbage supplement
Mortality,%	77(81)[1]	44(88)
Avg. Survival Time, days	18.6(62)	20.6(39)
Survivorship, ST_{50}[2]	19	38+
Spleen Wt/Body Wt × 100	.46(55)	.41(63)

1. No. of observations upon which value is based.
2. ST_{50} is no. of days until 50% of group is dead.

tassium acetate 2.7, magnesium oxide 0.5, DL-methionine 0.2, ascorbic acid 0.2, choline Cl 0.1, chlortetracycline HCl 0.0025 and the vitamin supplement previously described(5). Chlortetracycline protects guinea pigs against certain infections but does not prevent salmonellosis(6). It was included in the diet in an attempt to reduce secondary infections.

After the animals were adjusted to the experimental conditions they were infected with *Salmonella typhimurium* by intraperitoneal injection of approximately 100,000 organisms. The animals were then weighed and observed daily for a 42-day period. The Salmonella organism was originally isolated from a naturally infected guinea pig and identified serologically. The inoculum was prepared by growing the organism, which had been stored on beads in a frozen state, in trypticase broth overnight, collecting by centrifugation and resuspending in sterile saline solution (0.15 M).

Results. A summary of the results of 5 trials is shown in Table 1. The mortality rate among animals fed the basal diet varied from 64 to 100% and averaged 77%. Variation among the trials was not greater than might be expected due to chance ($X^2 = 1.24$; $P>0.8$). Mortality among the cabbage supplemented animals ranged from 27 to 54% and averaged 44% ($X^2 = 2.16$, $P> 0.7$). The difference in mortality due to cabbage supplementation (33%) was highly significant ($X^2 = 18.2$, $P<0.01$). There was no significant difference in mean survival time of the animals that died but the time required for one-half of the animals to die (ST_{50}) was more than twice as long for the supplemented group. Since in all but one trial (23 days) 50% of the animals fed cabbage did not die during the 42-day experimental period it is not possible to assign an accurate ST_{50} for this group. It can be stated that the period averaged greater than 38 days. Spleens and livers of all infected animals were fibrotic, greatly enlarged and contained viable organisms.

In one trial animals that survived the experimental period were carried for a total of 5 months. Of those fed the basal diet 50% survived and only one-fourth showed gross evidence of spleen or liver damage. Of the supplemented group 75% survived, and one-half showed organ damage. Thus, it appears that while the cabbage supplement increased the resistance of the animals it did not prevent or decrease the gross tissue damage caused by the infection.

Clearly cabbage supplementation of guinea pigs fed the basal diet decreased mortality rate, but neither the identity of the factor supplied nor the mechanism of its action is known. The basal diet contains adequate quantities of all the recognized vitamins and amino acids known to be required by the guinea pig, including an abundant supplement of ascorbic acid. When raw cabbage was supplied *ad libitum,* guinea pigs consumed about 25% of dry matter from cabbage and the remainder from the basal diet. Cabbage dry matter contains approximately 20% of crude protein and 2.5% of sulfur amino acids. Thus, substitution of cabbage for basal diet would tend to decrease protein but slightly increase sulfur amino acids. In growth trials carried out along with this study and to be reported later, additional methionine did not stimulate growth rate whereas cabbage supplementation did. For these reasons, cabbage is believed to contain an unrecognized nutrient required by the guinea pig.

Summary. Guinea pigs fed a purified diet with and without *ad libitum* supplementation with cabbage were inoculated intraperitoneally with *Salmonella typhimurium.* Over a 6-week period the supplement decreased mortality significantly and more than doubled survivorship. There was no effect on tissue damage among survivors. An unrecognized nutrient that increases resistance to salmonellosis is postulated.

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Anti-estrus Drugs on Subestrus of Ovariectomized C_3H Mice.* (26982)

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Gonadectomy in certain strains of mice leads, in time, to development of tumors of the adrenal cortex which secrete estrogen and/or androgen(1,2 and others). There is considerable evidence that these tumors develop as a result of the increased secretion of gonadotropin following castration. Nodular hyperplasia of the adrenal cortex of an intact mouse may be produced by parabiosis with a spayed partner(3). Estrogen, as well as certain other steroids(4), and hypophysectomy(5) will prevent their occurrence. Cortisone, in amounts sufficient to cause adrenal atrophy, does not prevent their development(6).

That formation of adrenal tumors and secretion of sex hormones from them may be dependent upon different mechanisms is shown by the findings that caloric restriction (7), thiourea administration(8) and unilateral adrenalectomy(9) prevent the appearance of subestrus but not the evolution of adrenal tumors. Yet secretion of estrogen by adrenal tumors may also be controlled by gonadotropin as evidenced by the observation that when an estrogen-secreting tumorous mouse is parabiosed with an intact mouse diestrus develops in the tumorous mouse, supposedly due to preferential utilization of gonadotropin by the ovaries of the intact mouse, but when a tumorous mouse is parabiosed with an ovariectomized mouse increased cornification in the vaginal smear of the tumorous mouse occurs due, supposedly, to the increased gonadotropin from the recently ovariectomized mouse acting on the adrenal tumor of its partner(10). On the other hand, administration of gonadotropin does not induce subestrus in tumorous mice on caloric restriction(11).

Inasmuch as reserpine, chlorpromazine, meprobamate(12), nidroxyzone(13), perphenazine and Enheptin† (2-amino-5-nitrothiazole) have been found to inhibit estrus in intact mice, a study of their effects on subestrus of ovariectomized mice was deemed worthwhile.

Methods and materials. C_3H female mice of the author's colony, originally obtained from Dr. J. J. Bittner, were ovariectomized and maintained on Purina Laboratory Chow. Drugs were administered in the diet after grinding with mortar and pestle and mixing with ground food. Control and drug-diets were fed *ad libitum,* except when otherwise noted. Vaginal smears were obtained by the lavage method. Mice were housed, usually 4 to 5 in a cage, in an air-conditioned, darkened-window room with automatic light cycles of 14 hours a day.

Mice were divided into 2 groups. Group I consisted of 60 animals ovariectomized between 8½ and 9½ weeks of age and placed on various drug-diets after subestrus was well established. Group II was composed of 88 mice ovariectomized between 9 and 14 weeks of age and put on drug-diets one week after ovariectomy.

A mouse was considered to be in subestrus

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† Unpublished data by the author.

TABLE I. Effect of Drugs on Subestrus Smears

Treatment	% Conc. in diet	No. mice	1st control period[a]			Treatm. per.		2nd cont. per[b]		Toxicity	
			Time[c]	No. days	% estrus[d]	No. days	% estrus	No. days	% estrus	No. died	% △ in body wt.
Reserpine	.0005	9	21	21	75.1	43	86.5	19	86.9	1[e]	+2.4
"	.00075[f]	7	26–30	14	85.7	28	70.4	27	50.8	0	—2.5
Chlorpromazine	.05[g]	6	24	17	85.7	39	69.2	27	34.6	0	+0.4
Perphenazine	.01–.005[h]	5	38	19	84.4	34	74.7	16	50.0	0[h]	—8.9
Meprobamate	2.0[i]	8	17–20	21	69.0	42	87.1	14	85.7	1[j]	—8.0
Nidroxyzone	.3	8	20	21	62.5	18–31[k]	61.6	—	—	8	—21.5
Enheptin	.4	8	20	21	60.1	62	58.5[l]	23	40.2	0	—15.4

[a] Immediately prior to treatment.

[b] " after "

[c] Weeks after ovariectomy.

[d] % estrus means % of total mouse days in which subestrus was noted.

[e] Died at 11 days. In addition, 2 died at 25 days due to lack of water.

[f] These mice had previously been on 2.0% meprobamate.

[g] These mice were on .1% chlorpromazine for 6 days 3 weeks previously. This concentration was toxic and 2 mice died.

[h] On .01% for 5 days, then on .005% for 29 days. One mouse died at 5 days, which is not included. These mice had previously been on reserpine (.0005%).

[i] 4.0% meprobamate to 8 other mice for 2 days proved toxic.

[j] Died at 24 days.

[k] All mice died, 18–31 days.

[l] During the last 42 days 5 mice had 68.6% estrus and 3 mice had only 18.3%.

when cornified cells were present in the smear. Leucocytes and nucleated epithelial cells were also always present, the latter appearing as single cells or clumped together. A diestrus smear consisted only of leucocytes plus or minus some epithelial cells.

Vaginal smears, taken periodically for 5 consecutive days during the first several weeks after gonadectomy in Group I, showed that subestrus had developed in an occasional mouse by 6 weeks and in most mice by 15 weeks post-ovariectomy. A general tendency toward cycling was noted but this was of a very irregular nature. Daily smearing (6 days a week) starting 17 to 23 weeks after ovariectomy was continued throughout the lifetime of mice in Group I.

Results. 1. Anti-estrus drugs on established subestrus. Group I mice were placed on drug therapy at 20 to 27 weeks after castration, preceded by 3 weeks of daily smearing while on control diet. Pertinent data are shown in Table I. It is apparent that none of the drugs used had any appreciable effect on estrus activity.

2. Anti-estrus drugs on development of subestrus. Group II mice were divided into 6 groups, one on ground control diet and 5 on drug-diets. Therapy, started one week after ovariectomy, was continued for 38 weeks, at which time surviving mice were autopsied. The data obtained from vaginal smears taken at various times are tabulated in Table II. The drugs used had no significant effect on vaginal smears at these times. The time of first subestrus noted varied from 26 to 117 days, with an average of 63 days for the entire group of mice, with no appreciable differences between the 6 sub-groups: control range, 39 to 74 days, avg. 61.5; reserpine, 41 to 74 days, avg. 63.8; chlorpromazine, 54 to 70 days, avg. 64.9; meprobamate, 42 to 95 days, avg. 63.0; nidroxyzone, 26 to 74 days, avg. 58.2, plus one at 117 days and one never showing subestrus; Enheptin, 33 to 74, avg. 60.5.

Body weights recorded weekly are summarized in Table III. During the first several weeks there were considerable variations in weights of treated mice due to changes in drug concentrations which were required in an effort to administer as high a concentration of drug as possible without excessively interfering with body growth. During the

TABLE II. Effect of Drugs on Development and Progression of Subestrus.[1]

Time after ovariectomy and effects		Control ground food	Reserpine .00025%	Chlorpromazine .05%	Meprobamate 2.0%	Nidroxyzone .15%	Enheptin .2%
5 wk:	No. estrus mice	2/15	1/13	0/15	2/15	3/15	3/15
	No. mouse days	75	65	75	75	75	75
	% estrus	2.7	1.5	0	2.7	5.3	5.3
9 "	No. estrus mice	15/15	12/12	15/15	14/15	12/14	15/15
	No. mouse days	75	60	75	75	70	75
	% estrus	60.0	66.6	56.0	74.7	70.0	67.3
13 "	No. estrus mice	12/15	11/12	9/13	15/15	9/13	14/15
	No. mouse days	180	144	135	171	156	180
	% estrus	27.8	31.9	34.8	49.7	32.7	46.7
20 "	No. estrus mice	10/15	10/12	9/11	7/14	10/13	13/15
	No. mouse days	75	60	55	70	65	75
	% estrus	28.0	36.6	32.7	38.6	49.2	52.0
27 "	No. estrus mice	11/12	9/12	9/11	8/11	10/11	12/14
	No. mouse days	300	300	263	275	232	350
	% estrus	38.0	13.7	19.0	15.3	18.9	22.9
36 "	No. estrus mice	9/12	7/12	3/8	7/10	3/5	7/11
	No. mouse days	156	156	104	127	67	143
	% estrus	21.2	15.4	19.2	31.5	29.9	23.1

[1] Vaginal smears were taken for 5 days at 5, 9 and 20 weeks, for 12 days at 13 weeks, for 25 days at 27 weeks and for 13 days at 36 weeks. No. estrus mice refers to the no. of mice showing subestrus at any time during the smear period, the numerator indicating number of mice showing subestrus and the denominator total number of mice surviving in the group. % estrus means % of total mouse days in which subestrus was noted.

first 10 weeks all treated groups weighed less than the controls, the chlorpromazine and Enheptin mice only slightly less, but the reserpine, meprobamate and nidroxyzone groups markedly so. In spite of this decrease in body weight of treated mice during the period of development of subestrus, no significant change in either onset or degree of estrus activity was noted.

3. *Waning of subestrus.* The data in Table II show that there was a tendency for subestrus to decrease after a period of time. Frequency in the different groups was 56 to 75% at 9 weeks, 28 to 50% at 13 weeks, 15 to 38% at 27 weeks, and 15 to 31% at 36 weeks. A similar decrease in subestrus of ovariectomized mice with time has been noted by Dr. Carlos Martinez‡ and is also apparent from the data in Table I.

4. *Effect of PMS in diestrus mice.* A group of 12 mice from Group I that had been in continuous diestrus for 7 to 80 days (avg. 28) while on control diet was given 2 I. U. of PMS subcutaneously per mouse without effect. A dose of 20 I. U. of PMS given 7 days later induced subestrus in 3 of the 12. These mice had been ovariectomized for 230 to 298 days.

5. *Effect of restricted food intake.* A group of 9 mice from Group I in frequent subestrus, also ovariectomized 230 to 298

TABLE III. Body Weights of Mice in Table II.

	Avg. body weights in g				
			Range during		
Group	Begin	10 wk	10-20 wk	20-30 wk	30-38 wk
Control	19.7	25.7	24.2-26.0	25.7-26.6	25.8-26.7
Reserpine	20.1	23.7	23.7-25.3	24.9-25.8	23.9-25.0
Chlorpromazine	20.6	24.9	24.2-26.4	24.9-26.6	23.8-25.2
Meprobamate	19.9	23.0	22.5-25.4	23.9-25.0	24.0-25.0
Nidroxyzone	20.1	22.7	21.0-23.2	22.5-24.3	21.9-23.4
Enheptin	20.0	24.6	23.1-24.6	23.0-24.4	22.0-24.1

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‡ Personal communication of unpublished data.

days previously, was given 2 g of control food per day per mouse for 35 to 72 days. The frequency of subestrus for the preceding 25 days of *ad libitum* control food (225 mouse days) was 53.3%, and for the entire restricted period (530 mouse days) was 48.7%. During the first 35 days of restricted intake frequency was 64.1% (9 mice), but during the last 20 days was only 21.0% (5 mice). Three mice died between 35 and 45 days and one was lost at 47 days. Of the 3 that died subestrus was present the day before death in 2, and 3 days before death in the third. Average body weight lost was 36.6% at 35 days and 40.8% at 72 days. Thus, restriction of over-all food intake had little effect on subestrus.

6. *Autopsy findings.* A total of 114 mice were autopsied. Three did not show gross evidence of adrenal tumors but had shown a high frequency of subestrus smears. In the remaining 111 mice white patches were grossly apparent in the adrenals. Microscopic examination of several adrenal glands by Dr. Carlos Martinez showed them to contain adenomas.

Discussion. In intact mice estrus cycles have been found to be decreased in ZBC mice by 74 and 84% with 0.00075 and 0.001% reserpine, by 47 and 70% with 0.2 and 0.3% chlorpromazine, by 61 and 89% with 2.0 and 3.0% meprobamate(12); in C_3H mice by 80 to 96% with 0.2% nidroxyzone(13) and by 73 to 95% with 0.1 to 0.2% Enheptin;† in ZA mice by 67 to 87% with 0.005 and 0.01% perphenazine.† These doses had only minor effects on body weight. In the present communication the concentrations of drugs used are sometimes less than the above but were approximately the maximally tolerated doses in ovariectomized C_3H mice. C_3H mice weigh less and generally tolerate less drug than the other strains used above. That these drugs inhibit estrus in intact mice but do not affect subestrus in ovariectomized mice suggests a difference in the mechanism for control of secretion of estrogen from ovaries of intact mice and of estrogen secretion from adrenals of ovariectomized mice, or, possibly, a difference in the type of estrogen secreted.

Administration of PMS had only a slight effect on the diestrus that developed in some mice many months after castration. This is in accord with the inability of gonadotropin to induce estrus reported by Martinez, *et al.* in ovariectomized, unilaterally adrenalectomized mice(9) and by King, *et al.* in caloric-restricted, gonadectomized mice(11).

The lack of effect of restricted food intake on frequency of subestrus was somewhat surprising for Casas *et al.*(7), and Ferguson *et al.*(5), found that food restriction prevented secretion of estrogen from adrenal tumors in C_3H castrated mice. These workers instituted food restriction at the time of gonadectomy whereas, in our work, mice had been castrated approximately 8 to 10 months before restriction was started. It may be that food restriction prevents the ability of adrenal tumors to start secreting estrogen but does not interfere with its secretion after initiation, due possibly to some effect on the functional, though not histological, development of cells intended to become estrogen-secreting.

Summary. Certain drugs, namely reserpine, chlorpromazine, perphenazine, meprobamate, nidroxyzone and Enheptin, previously found to have anti-estrus effect in intact mice, did not alter the frequency of subestrus nor development of subestrus in ovariectomized C_3H mice. Other findings in ovariectomized mice were: (1) frequency of subestrus tended to decrease with time and (2) restriction of food intake in mice with established subestrus did not alter frequency of subestrus.

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Study of Copper and Zinc Metabolism During Pregnancy. (26983)

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Previous studies have demonstrated that the total serum copper level increases with administration of certain hormones such as testosterone and estrogen, during pregnancy, and in different pathological states(1,2,3,4). Few studies have been reported on the effect of sex hormones on serum zinc level. The studies in this laboratory indicated that total serum zinc level declined with parenteral administration of testosterone propionate and estradiol benzoate. The mechanism of the mobilization and utilization of copper and zinc with the endogenous release or administration of sex hormones is unknown. The purpose of this investigation was to determine if total serum zinc level is changed during pregnancy.

It is known that the zinc content in serum is greater than the copper content. We found that the normal serum copper and zinc ranged from 99-113 μg/100 ml and 158-178 μg/100 ml, respectively, on 200 geriatric patients(4), as compared to 84-137 μg/100 ml and 158-285 μg/100 ml reported by others on middle aged subjects(5,6,7).

Method. Thirty female subjects were selected for this investigation. Their ages ranged from 21-40. All subjects were ambulatory with no acute or debilitating disease, and with no known disorder of copper or zinc metabolism. Twenty subjects were pregnant from 12-40 weeks. Ten non-pregnant subjects were used as controls. Venous blood was secured with the necessary precaution to avoid contamination. Duplicate determination of total serum copper and zinc was made during pregnancy and 8 weeks post partum. Colorimetric methods were employed for trace mineral determination(8,9) with modification by the author. Serum oxidase activity was determined by the method of Ravin(12), modified by Houchin(13).

Results: Table 1 gives the code, ages, weeks of pregnancy, total serum level of zinc, copper, and oxidase activity during the indicated period of pregnancy and 8 weeks after parturition. While the serum zinc level was found to be lower during pregnancy in all subjects, the serum copper level was increased in all subjects. The lowest zinc value and highest copper value were 45 and 289 μg/100 ml, respectively, for a subject 29 weeks pregnant. Eight weeks after parturition we found that the copper value decreased and the zinc value increased, approaching the normal reported range. The copper oxidase activity value ranged from 0.26-0.37 OD unit during pregnancy and declined after delivery to a range of 0.13-0.27 OD unit.

Discussion. Several investigators have observed an increase in total serum copper values in pregnancy and estrogen administration(1,5,6,4) and one author(10) has reported a decline in zinc during pregnancy. The results of investigation on copper and zinc excretion(1,5,11) during pregnancy and sex hormone administration indicated such changes are not significant. A high degree of correlation was observed between total serum copper and oxidase activity(14) in

TABLE I. Total Serum Copper and Zinc Levels and Oxidase Activity Before and After Parturition

			Before parturition			8 Weeks postpartum		
Code	Age	Wks. of Pregnancy	Zn μg	Cu %	Cu Oxidase O.D.	Zn μg	Cu %	Cu Oxidase O.D.
C-2	32	12	175	170	.35	195	110	.27
T-1	27	16	55	205	.26	201	150	.22
C-5	32	20	115	224	.33	199	116	.20
C-6	31	20	110	214	.37	206	120	.20
T-3	30	32	153	259	.30	212	114	.18
C-4	34	23	82	180	.33	175	128	.28
W-4	23	29	45	289	.32	188	121	.23
C-3	31	30	118	186	.30	194	100	.19
C-1	27	30	90	245	.31	185	145	.16
T-4	27	30	148	259	.31	210	125	.18
W-7	27	33	80	256	.32	257	126	.23
W-8	35	35	45	197	.34	310	117	.25
W-9	30	36	58	275	.33	240	128	.27
W-5	25	36	63	283	.31	125	82	.20
W-6	31	36	95	227	.34	205	115	.24
T-2	24	38	113	207	.29	220	101	.16
W-1	38	39	63	275	.31	205	98	.17
W-10	21	39	65	255	.35	213	105	.26
W-3	30	40	75	289	.34	222	120	.26
W-2	34	40	50	179	.28	207	110	.13
10 control subjects			192	116	.19	197	114	.19

Age range in control subjects from 21 to 28.
Second determinations on control subjects were done 8 weeks after the first.

pregnant women. Serum copper and zinc levels tend to increase and decrease respectively with administration of sex hormone in non-pregnant subjects, and in pregnant subjects where there is known to be an increase in sex hormone. These facts suggest the changes are due to the change in hormone level in the body.

Summary. 1. There was an increase in total serum copper content and a decrease in total serum zinc content during pregnancy. 2. The total values of copper and zinc returned to or near their normal reported range, post partum.

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Relation Between Accommodation and Minimal Gradient.* (26984)

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It is well recognized that tissues have a minimal gradient requirement (lambda)(1,2) which must be satisfied if applied pulses are to stimulate nerve, cardiac or skeletal muscle(2,3). Current pulses which have too slow a rise time do not stimulate, supposedly because accommodation has occurred(2). The term accommodation is also used to describe the decrease in facilitatory effects of long lasting subthreshold conditioning pulses(4). The terms catelectrotonus and anelectrotonus are well known to physiologists and it is generally recognized that the effectiveness of the applied current decreases somewhat because of accommodation. The purpose of the present paper is to give evidence indicating that it is unsafe to assume that "accommodation" is the same in both cases.

Method. Dog ventricle trabecular muscles were used in this work. They were maintained in a tissue bath and perfused with Tyrode's solution. Rectangular pulses and pulses with a controlled rate of linear rise were used in determining thresholds and minimal gradient requirements. Long duration conditioning pulses could also be applied. The methods of such studies have been described fully elsewhere(3) but Fig. 1 gives a diagram of the devices employed.

Results. Fig. 2 shows the changes in threshold to testing stimuli which occurred during and following application of a 280 msec conditioning pulse of half-rheobasic strength. It can be seen that an initial facilitatory action was not fully sustained. Threshold rose slightly during the first 100 msec of the cathodal current flow and then remained relatively constant until break of the current. The postcathodal rise in threshold which lasted somewhat more than 50 msec is the typical postcathodal depression. This decrease in facilitatory action of the conditioning pulse is the accommodation referred to and the experiment demonstrates that the phenomenon does occur in this type of cardiac tissue.

It has been recognized for some time that slowly rising pulses excite when they reach a rheobase determined by rectangular testing pulses(5,6). This in itself gives some indication that accommodation, in the sense of a rise in threshold during continuing current flow, does not occur. However, it seemed reasonable to test the possibility of accommodation to linearly rising currents by applying test pulses at various intervals after commencement of the current. Fig. 3 presents the result of an experiment in which a linearly rising pulse that just failed to stimulate was used as the conditioning current flow. In (A) the upper tracing shows a pulse of rheobasic strength and the lower tracing the response evoked. In (B) is shown a pulse which rose to rheobasic strength at too slow a rate to stimulate. In records C to H the minimal intensities of applied short-duration pulses which stimulated the muscle are indicated by an arrow. No adaptive or accommodative change occurred since it was found that addition of conditioning and test pulse strengths came to the same total during the rising phase of the "conditioning stimulus." At the break of this conditioning pulse, however, threshold requirements rose.

Fig. 4 was made by superimposing a series of tests such as were portrayed in the preceding figure. The conditioning pulse is shown in the lower tracing. The upper line shows the threshold as determined by addition of testing and conditioning pulse. Again it is seen that since the sum of the 2 did not change, threshold did not change and there is no evidence of accommodation. The postcathodal depression, or threshold elevation, however, is nicely demonstrated.

Whatever the "accommodation" shown in Fig. 2 may be, it certainly does not explain minimal gradient requirements. The process

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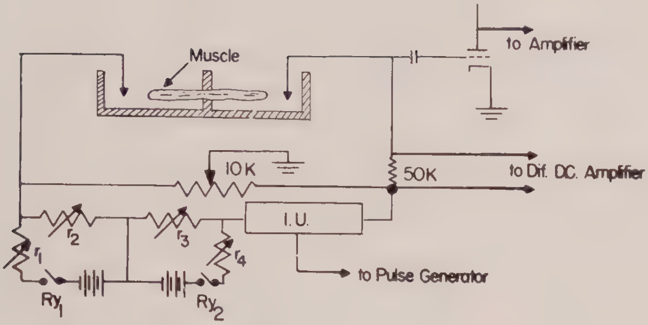

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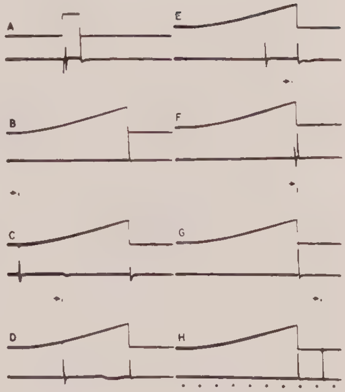

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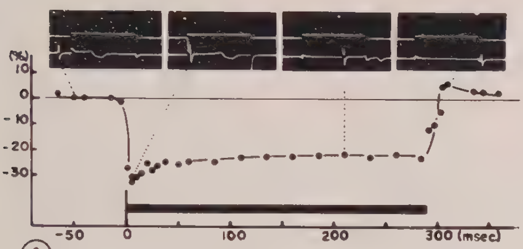

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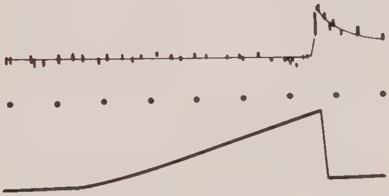

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FIG. 1. Experimental arrangement for testing accommodation and minimal gradient in perfused muscle bundle.

FIG. 2. Showing accommodation to conditioning pulse (0 to 280 msec). Threshold (•——•) shown in percentage of normal. Upper records show the applied pulses and the resulting response (spike potential).

FIG. 3. Determination of threshold (→) by summed linearly-rising and test pulses. Lower trace shows evoked response (spike potential). Time intervals 100 msec.

FIG. 4. Diagram drawn from superimposed tests. Lower tracing an ineffective linearly-rising pulse. Upper tracing shows thresholds to testing pulse alone or summed pulses. Middle dots show time intervals of 100 msec.

which is responsible for the failure of slowly rising currents to stimulate can be called accommodation but it differs from the failure of facilitatory action seen when conditioning and testing currents are paired as in Fig. 2.

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Protection Against *Naja naja* Venom in Dogs by Hydrocortisone.* (26985)

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Although utilized clinically in therapy of ophidism, the value of steroid treatment has not been clearly defined. Experiments recorded here were designed to determine if steroid treatment would favorably alter the outcome following administration of venom of the cobra, *Naja naja,* in lethal dosage to dogs. Results indicate that when this substance is given in massive doses at frequently repeated intervals, survival of some animals can be obtained.

Method. Adult mongrel dogs of either sex were anesthetized with sodium pentobarbital (15 mg/kg). Arterial blood pressure was measured by means of a pressure transducer and Sandborn recorder utilizing a plastic catheter in the femoral artery. A large bore plastic tube was passed into the trachea of the animal and respiration maintained by a mechanical respirator utilizing room air. The calculated dose of dried venom of *Naja naja* reconstituted with 1-2 ml of normal saline was administered intravenously. Treated animals received hydrocortisone intravenously and no other medication or therapy was utilized. Hydrocortisone was given before venom injection or following it and was repeated when systemic arterial pressure began to fall. Animals were treated until blood pressure appeared to be stabilized or proved refractory to further injections of the steroid. After periods of 4 to 12 hours of observation and treatment, the arterial cannula was removed from the femoral artery of survivors and the artery ligated. Artificial respiration was terminated and the animals were returned to their cages. Survivors were classified as those animals living at least 10 days beyond the experiment.

Results. Sixteen control animals received from 1 to 3 mg of cobra venom/kg body weight intravenously. Immediately following venom administration there was a precipitous fall in arterial blood pressure followed by its recovery to near normal levels. Thereafter arterial pressure fell slowly until a preterminal phase was reached where a precipitous drop occurred just before death. Duration of survival of animals is shown in Table I. All animals died. Three animals receiving 1, 1.25, and 1.5 mg of venom/kg body weight lived 8, 12 and 16 hours respectively. All others died within 4 hours of time of venom injection.

Table II shows that pretreatment alone with 15-30 mg hydrocortisone/kg did not result in survival of any animals. However, pretreatment followed by additional hydrocortisone administration in similar massive doses after envenomation resulted in survival of all 3 animals so treated. When hydrocortisone was administered only after venom injection survival of 3 of the animals receiving 2 to 2.5 mg of venom/kg body weight occurred (Table III). Although they eventually died, life was prolonged in at least 3 other dogs.

Discussion. Several authors have recorded favorable clinical impressions in treatment of

TABLE I. Length of Survival of Dogs Following Intravenous Injection of Venom of *Naja naja*

Animal	Wt (kg)	Venom mg/kg	Period of Survival
1	12.3	1	8 hr.
2	9.5	1.5	47 min.
3	6.8	1.25	12 hr.
4	10	1.5	99 min.
5	8.6	1.5	16 hr.
6	12.3	1.65	160 min.
7	10	2	115 "
8	10	2.25	51 "
9	11.8	2	71 "
10	10.5	2.30	42 "
11	7.2	2.30	51 "
12	9.1	3	60 "
13	7.6	3	42 "
14	10.5	2.5	240 "
15	9.1	3	180 "
16	8.6	3	125 "

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bites by copperheads, vipers, and cobras with steroid.(1-5) Experimental data are more in conflict. No significant effect of cortisone on mortality rates following injection of a lethal dose of *Crotalus adamanteus* venom could be shown by Allam and associates(6). However, Deichman and associates(7) found hydrocortisone to protect some of the dogs studied from "two approximate lethal doses" of *Crotalus adamanteus* venom when the steroid was given intravenously immediately or 2 to 4 hours after the venom. Schottler(8) reported that ACTH, cortisone or hydrocortisone afforded no protection for mice injected with

TABLE II. Treatment of Experimental *Naja naja* Envenomation in Dogs with Hydrocortisone.

Dog	Wt (kg)	Venom mg/kg	Hydrocortisone mg/kg	Time given	Period Survival
17	9.1	2	30	Pretreated (45 minutes)	4 hr
18	8.2	3	30	idem	22 min.
19	7.2	3		Pretreated with hydrocortisone 15 mg/kg a day × 5 days	125 “
20	8.2	2	30	Pretreated (30 min)	Survival
			15	4 hr after venom	
21	7.6	2	15	Pretreated (60 min)	“
			30	30 min after venom	
22	7.2	2	30	Pretreated (30 min)	“
			20	3 hr after venom	
			15	7 hr after venom	

TABLE III. Treatment of Experimental *Naja naja* Envenomation in Dogs with Hydrocortisone.

Dog	Wt (kg)	Venom mg/kg	Hydrocortisone mg/kg	Time given	Period Survival
23	11.4	3	30	20 min after venom	5 hr 10 min
			15	4 hr after initial dose	
			15	4 hr “ 2nd dose	
24	11.8	2	30	30 min after venom	Survival
			15	4 hr after venom	
			15	8 hr “ “	
25	9.1	2	30	1 hr “ “	“
			15	5 “ “ “	
26	7.2	3	40	45 min after venom	5 hr 15 min
			20	4 hr “ first dose	
27	6.3	3	40	45 min after venom	26 hr
			40	4 hr “ initial dose	
			40	4 “ “ 2nd dose	
28	9.1	3	40	20 min after venom	20 hr
			40	4 hr “ initial dose	
			40	4 hr “ 2nd dose	
			30	12 hr “ venom	
29	10.5	2.5	40	30 min after venom	5 hr 30 min
			30	4 hr “ initial dose	
30	8.1	2.5	40	30 min after venom	Survival
			30	4 hr “ initial dose	
			20	4 hr “ 2nd dose	
			20	4 hr “ 3rd dose	
31	9.1	2.5	40	25 min after venom	3 hr 30 min
32	9.1	2.5	40	40 “ “ “	12 hr
			30	4 hr “ initial dose	
			30	4 “ “ 2nd dose	
33	10.5	2	40	30 min after venom	3 “

LD_{50} of *Crotalus terrificus* or *Bothrops jararaca* venom. Russell and Emery(9) found methylprednisone and hydrocortisone did not suppress the lethal activity of *Ancistrodon contortrix* venom in mice with the possible exception that hydrocortisone in dosage of 100 mg/kg body weight afforded some protection. Ganatra and associates(10) stated that hydrocortisone in dosage of 2 mg/20 g body weight significantly reduced the mortality rate of mice injected with 2 LD_{50} of viper venom as compared to antivenin. These authors commented that the doses of hydrocortisone used in this experiment were very large. There are no previously reported experiments wherein the effect of hydrocortisone on experimental cobra envenomation has been studied.

The mechanism of the lethal effects of venoms and bacterial endotoxin appear in many respects to be similar(11). Lillehei and MacLean(12) found pretreatment with hydrocortisone in massive doses protected the majority of dogs tested against endotoxin in lethal dosage. In the experiments reported here, doses of hydrocortisone utilized were much greater than those usually employed clinically. Repetition of hydrocortisone administration whenever systemic blood pressure began to fall appeared necessary for survival of animals. In preliminary experiments not recorded here, when smaller amounts of cortisone or hydrocortisone were utilized, no apparent benefit was obtained and all animals died. As in the experiments with endotoxin (12), it appears that massive doses of the substance are necessary if animals are to survive.

Summary. Hydrocortisone in large doses reduces the lethality of *Naja naja* venom for dogs.

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Multiplication and Cytopathogenicity of Mouse Hepatitis Virus in Mouse Cell Cultures.* (26986)

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Since 1951, several investigators have reported isolation of 8 viral agents which produce a focal hepatic necrosis in mice(1-8). Spontaneous disease was induced by none with the exception of the virus of Gledhill and Andrewes(1). The remainder became manifest only in mice inoculated with tissue suspensions in attempts to pass other agents. A number of differences between certain of these agents have been demonstrated which suggest the group is heterogeneous. Never-

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theless, Morris and Aulisio(7) were unable to reveal antigenic differences between the 4 strains which they studied. Additional comparative researches are clearly needed to define the relationships between these viruses.

No convenient method whereby they can be studied *in vitro* has been available. Although the strain MHV_1 has been propagated in tissue culture(9,10), no cytopathic effect (CPE) was observed. For infectivity assay, therefore, even with this strain mice were still required. Miyazaki, *et al.*(11) propagated MHVB (Buescher) in mouse liver tissue culture for 94 passages, but were able to detect viral replication only by comparative cell counts of infected and control cultures. Bang and Warwick(12) alone have observed CPE by mouse hepatitis virus. These workers found that MHV(P) strain destroyed macrophages from liver tissue grown on a collagen substitute.

Investigations to be reported here on the behavior *in vitro* of the Balb C strain(8) of virus were initiated with the purpose of developing a more convenient procedure for viral and serological assay.

Methods and materials. Virus. Balb C strain of MHV was kindly supplied as a lyophilized suspension of infected mouse brain by Dr. John B. Nelson. Stock virus was prepared in this laboratory from mouse livers of the second intraperitoneal passage. Mice were sacrificed on the 4th day and livers ground in a chilled mortar. A 10% suspension in Hanks' balanced salt solution (BSS) was passed through a Seitz S3 filter, distributed in ampoules, quickly frozen, and stored in a dry-ice chest. During storage no loss in mouse infectivity was observed over a period of 10 months.

Mice. The Harvard strain of Swiss mice was used for all experiments. Spontaneous hepatitis has not been observed in these animals.

Mouse embryo tissue culture. Fourteen to 16-day old mouse embryos were dispersed as a cell suspension in 0.25% trypsin in Hanks' BSS at 37°C for 1 hour. After centrifugation they were resuspended in medium 199 with 2% inactivated (56°C) calf serum and dispensed in tubes. Infection was usually carried out on the 3rd day. Medium 199 with 1% inactivated calf serum was used for maintenance.

Mouse kidney tissue culture. Monolayers of kidney epithelium from newborn and adult mice were kindly supplied by Sumner Berkovich. These were prepared by a modification of the method of Youngner(13) for monkey kidney cell cultures and were grown in 0.5% lactalbumin hydrolysate in Hanks' BSS with 10% inactivated calf serum. The same medium with 5% calf serum was used for maintenance.

L cells. Most experiments were carried out using L cells supplied by Dr. R. S. Chang that were grown and maintained in Eagle's minimal essential medium with 10% horse serum. Results were confirmed with another lot of L cells supplied by Dr. Monroe D. Eaton. For titrations that included 3 or 5 cultures for each dilution ten-fold serial dilutions of virus were prepared. The volume of inoculum was 0.1 ml. Incubation was at 37°C in a roller drum. Cultures were observed daily for cytopathic effect.

MHV antibody. The method of Kasel *et al.*(14), as modified by Berkovich(15) for production of viral antibodies in mouse ascitic fluid was employed. Five adult Swiss mice were inoculated intraperitoneally with 0.1 ml of a 10^{-3} dilution of the brain suspension supplied by Dr. Nelson. One of the animals died. Ten days later the remainder were inoculated with undiluted virus. Five control mice were injected with Hanks' BSS at the same time. Seven days after the second injection approximately 10^7 Ehrlich's ascites tumor cells were introduced intraperitoneally in both groups. Ten days later the serosanguinous ascetic fluid was harvested, centrifuged and stored at —15°C as the antibody solution.

Results. Viral multiplication in mouse embryo tissue culture. Mouse embryo monolayer cultures (METC) were inoculated with 0.1 ml of MHV (Balb C) as a 10% liver suspension. Undiluted culture medium harvested on the 7th day, when introduced intraperitoneally in weanling mice was followed by death with gross hepatic damage. Serial propagation in METC was then undertaken, and carried through 15 passages. Death with liver damage of intraperitoneally inoculated

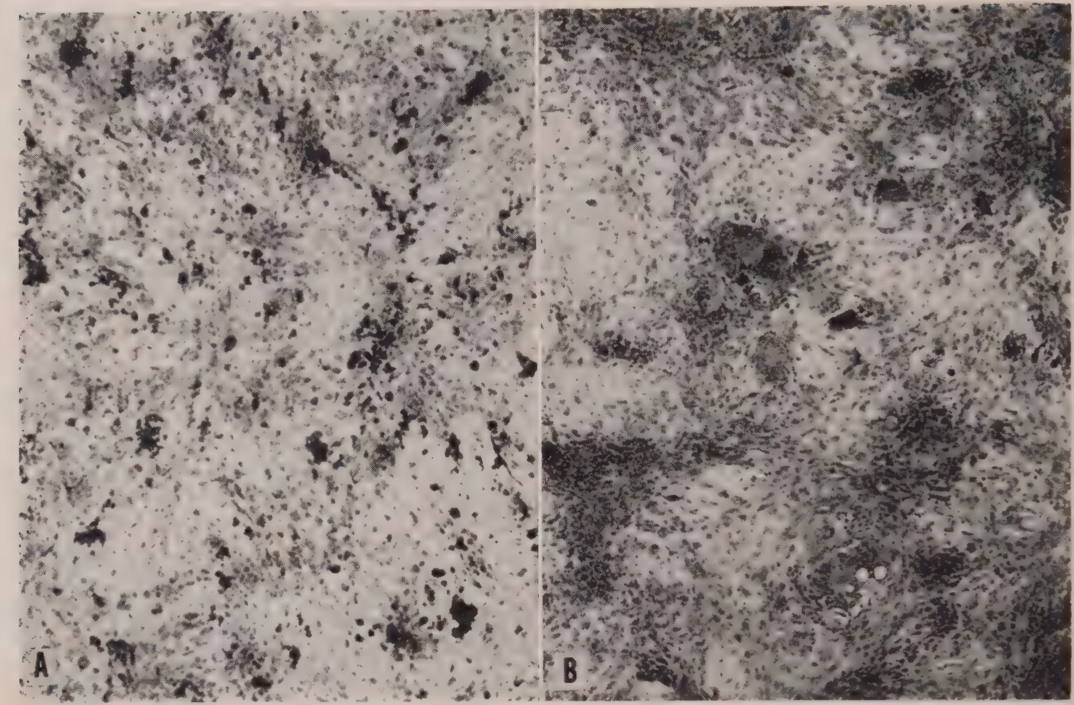

FIG. I. Changes in mouse embryo tissue culture infected with mouse hepatitis virus. Hematoxylin and eosin stain.

a. Uninfected METC. Magnification × 61.

b. METC 72 hrs after infection with MHV, 14th passage. Magnification × 61.

weanling mice was observed with medium harvested from the 3rd, 7th, 11th, and 15th tissue culture passages. Mouse infectivity titer of the liver suspension used to initiate the first passage was $10^{-2.5}LD_{50}$/ml. Titer of fluid from the 15th cell culture passage was $10^{-5}LD_{50}$/ml. Since the original inoculum in this passage had been diluted more than 10^{-20}, replication of the virus in tissue culture was demonstrated.

Histopathologic lesions in mice produced by the original virus and the 15th METC passage were identical and consisted of the same type of focal necrosis with scant cellular reaction.

To determine whether the agent present after 2 mouse and 15 METC passages was antigenically identical with that in the original seed a neutralization test using ascitic fluid from mice immunized with the seed virus was carried out. A 1:10 dilution of heated immune ascitic fluid neutralized $10^{3}LD_{50}$ of MHV while a similar dilution of heated ascitic fluid from control animals failed to neutralize $10^{2}LD_{50}$. It is concluded therefore that the agent propagated is antigenically similar to the Balb C strain.

Cytopathic effect in METC was not observed until the 7th passage when vacuolization, eosinophilic cytoplasmic inclusions, and multinucleate giant cell formation were noted. The extent of these changes in the several subsequent passages varied, but they were easily and consistently recognized following the 11th passage.

The cytopathic effect in METC of the agent in the 14th passage is illustrated in Fig. I. By 48 to 72 hours giant cells are present which exhibit a central area of homogenous, intensely eosinophilic masses surrounded by 5 to 100 nuclei within a cytoplasmic sheet which is frequently vacuolated. Many cells are subsequently lost from the sheet, but are partly replaced by secondary growth. These changes are not followed by complete destruction of the cell sheet which survives for approximately the same length of time as in uninoculated cultures. As incubation is continued giant cells become fewer and the cell sheet appears to be less extensively involved. Giant cells have been observed, however, as late as 28 days after infection.

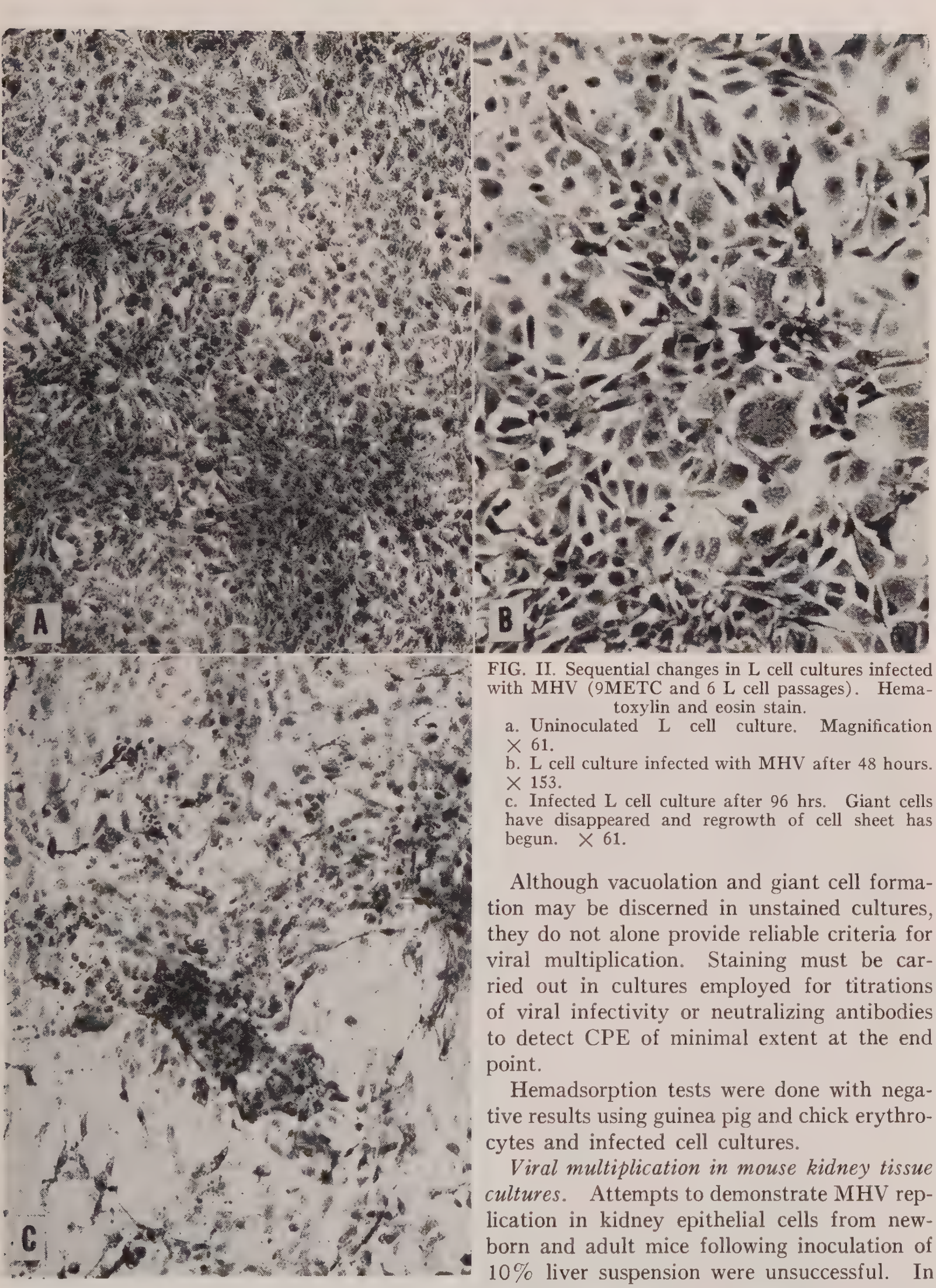

FIG. II. Sequential changes in L cell cultures infected with MHV (9METC and 6 L cell passages). Hematoxylin and eosin stain.
a. Uninoculated L cell culture. Magnification × 61.
b. L cell culture infected with MHV after 48 hours. × 153.
c. Infected L cell culture after 96 hrs. Giant cells have disappeared and regrowth of cell sheet has begun. × 61.

Although vacuolation and giant cell formation may be discerned in unstained cultures, they do not alone provide reliable criteria for viral multiplication. Staining must be carried out in cultures employed for titrations of viral infectivity or neutralizing antibodies to detect CPE of minimal extent at the end point.

Hemadsorption tests were done with negative results using guinea pig and chick erythrocytes and infected cell cultures.

Viral multiplication in mouse kidney tissue cultures. Attempts to demonstrate MHV replication in kidney epithelial cells from newborn and adult mice following inoculation of 10% liver suspension were unsuccessful. In contrast inoculation of these systems with virus derived from a single passage in METC resulted in multiplication in mouse kidney epithelial cells with associated, although minimal CPE, essentially similar to that in METC.

Viral multiplication in L cells. Addition of the virus in the form of 10% liver suspension or the fluid from a single passage in METC to cultures of L cells was followed by loss of the agent as determined by inoculation of weanling mice. Harvest from the third METC passage did survive or multiply in L cells. Following the 15th direct METC passage, the virus induced CPE upon first passage in L cells.

Fig. II illustrates sequentially the changes that occur in stained L cell cultures. Eosinophilic intracytoplasmic masses and multinuclear giant cell formation are present, as well as focal degeneration and cell depletion. Four to 6 days after infection giant cell formation becomes scanty and regrowth of the cell sheet begins. CPE is easily recognized in unstained L cell cultures (Fig. III). It provides a reliable criterion of viral activity in titrations of infectivity and in neutralization tests. The changes are maximal by the 5th day at the titration end point and by the 3rd day with a larger inoculum.

The 15th METC passage gave a titer of $10^{-5.2}ID_{50}$/ml in L cells as compared with a titer of $10^{-5.3}LD_{50}$/ml in suckling mice.

In Vitro neutralization. Neutralization of CPE in L cell cultures by mouse MHV immune ascitic fluid was demonstrated. Inactivated immune fluid prevented CPE in a dilution of 1:128. Higher dilutions were not tested. Unheated immune fluid exhibited the same neutralizing capacity. Control ascitic fluid inhibited CPE only in a dilution of 1:4.

Growth curves. To determine the characteristics of MHV multiplication in tissue culture growth curves were carried out in METC and L cell cultures. Virus carried through 9 METC and 6 L cell passages was used in these experiments. Results are summarized in Fig. IV. Maximal titer as determined in L cell cultures was obtained in METC on the 3rd day, but a constant production of virus was maintained over a period of 28 days. In L cells a chronic infection was also established although virus after an initial increase dropped for a time to an un-

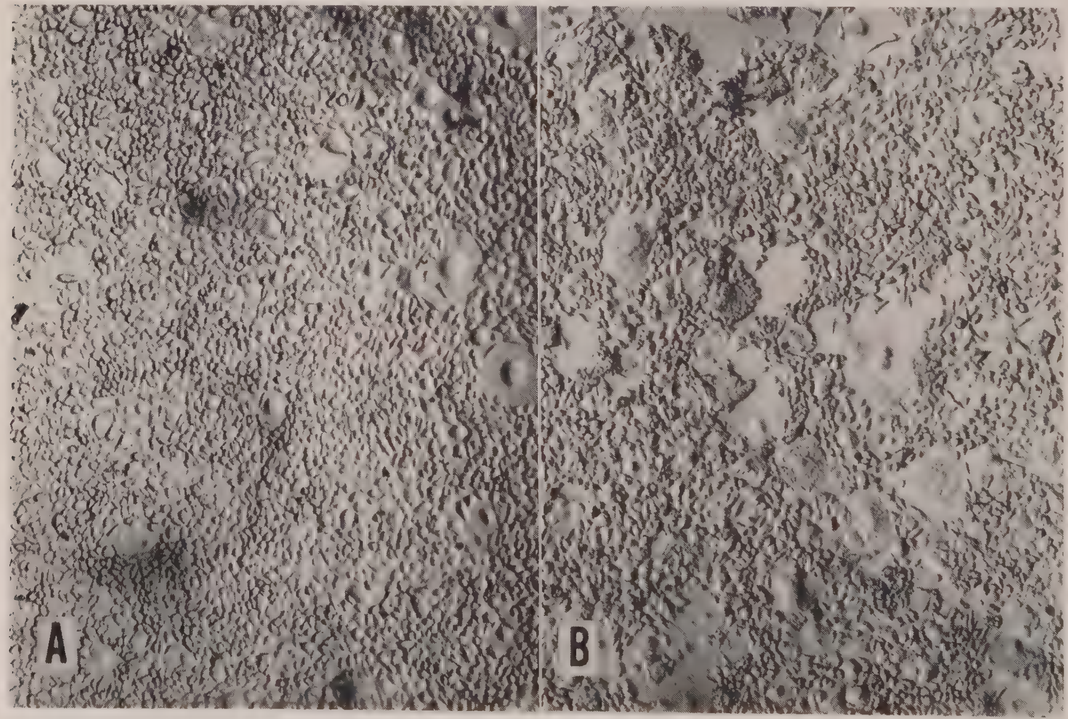

FIG. III. Unstained L cell cultures. Magnification × 61.
a. Uninfected.
b. 72 hrs after infection with MHV, 15 METC passage × 61.

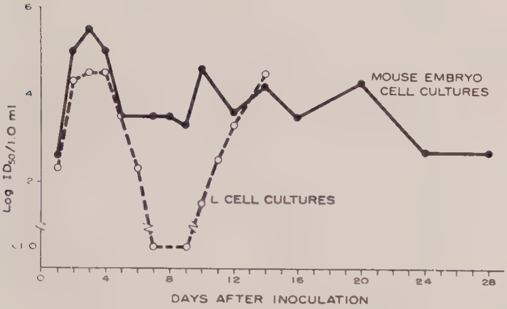

FIG. 4. Growth Curve of Mouse Hepatitis Virus in Mouse Embryo and L Cell Cultures as Determined by Assay in L Cell Cultures

detectable level only to reappear after the cell sheets had again grown out.

Discussion. Interest in mouse hepatitis viruses has derived largely from the activation of such latent agents in the attempt to transmit human hepatitis and leukemia. The discovery of these agents has also afforded a convenient model for laboratory study of problems relating to a viral hepatitis such as the effect of diet(16) and of the mechanism of transaminase elevation(17). The availability of a convenient *in vitro* method for detection of at least one member of the group should facilitate such studies.

It is of interest that a single passage of the virus under study in a susceptible cell system rendered it capable of multiplication in another system consisting of cells in which initially replication did not occur. Further investigation of this phenomenon seems desirable as the results may suggest procedures whereby adaptation of virus to an initially resistant cell can be accomplished.

Summary. The data presented indicate that MHV (Balb C) multiplies in mouse embryo cell cultures and, after passage in that system, in mouse kidney epithelium and L cells. In all these systems a cytopathic effect was observed after serial passage. These observations provide convenient techniques for studying *in vitro* this and perhaps other strains of mouse hepatitis virus.

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Urinary Mucopolysaccharide Excretion in the Sex-Linked Form of the Hurler Syndrome.* (26987)

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Hurler's syndrome (lipochondrodystrophy, gargoylism) is an hereditary aberration of the metabolism of mucopolysaccharides in human

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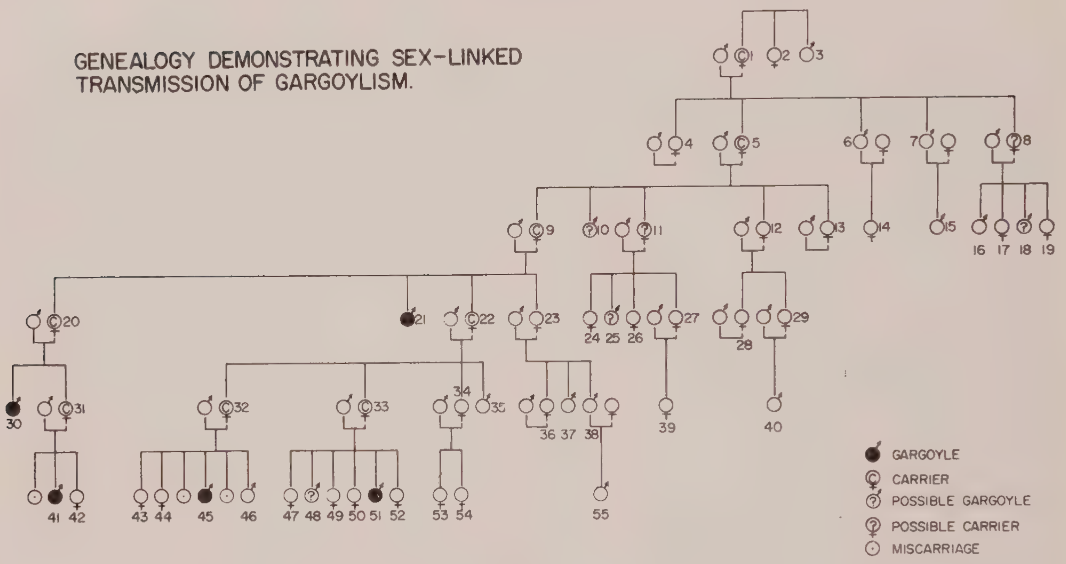

FIG. 1

beings, characterized by grossly defective mental and physical development and early, usually prepubertal, death(1). In the majority of the cases described in the literature, the disease is transmitted as an autosomal recessive trait although partial expression may occur in heterozygotes. There is also a somewhat rarer sex-linked mode of transmission in which the disease is usually manifested later in life, is restricted to males, and would appear to be clinically less severe.

Brante(2) reported that the Schiff-periodate staining material in the tissues of gargoyles was a complex acid mucopolysaccharide containing hexosamine, sulfate, and uronic acid moieties. Excretion of mucopolysaccharides in the urine of gargoyles in quantities much greater than normal has been described (3,4). The excreted material is largely composed of chondroitin sulfate B and heparin monosulfate. Although at least 2 mucopolysaccharides of differing structure and composition are affected by the disease these 2 classes of compounds do have some structures and precursors in common(5), and it is possible to postulate that a single enzymatic defect underlies the complex symptomatology.

The present study was designed to develop methods for the determination of mucopolysaccharide levels in urine and to apply them to members of a family in which the sex-linked form of gargoylism was encountered. The family is similar to that described by Beebe and Formel(6) although none of the affected individuals have lived to maturity. The possibility of using chemical methods for detecting carriers of the trait was also investigated. A preliminary report of this work has appeared(7).

The genealogy of the family studied is shown in Fig. 1. Individuals from 4 generations have been available for testing. Information about others was derived from photographs, family Bibles, and cross-checked interviews with surviving members of the family. It is a genealogy typical of the transmission of a sex-linked trait. Almost exactly 50% of the females have been carriers and nearly 50% of the males have been clinically affected. There have been no clinically affected females. There are 3 gargoyles in the most recent generation and 2 well authenticated cases in the preceding 2 generations. The genealogy indicates that 7 females, not including the first generation, have been carriers. It is not known whether the trait arose as a mutation in #1 or #5 or was present in the family at an earlier time.

Experimental. Twenty-four hour urine samples were obtained from all of the available members of the family in the direct hereditary line. Aliquots of the urine were dialyzed exhaustively against running distilled water in the cold and diluted 1:1 with distilled water. This dialyzed and diluted urine was then analyzed for total mucopolysaccharide by the following methods.

1. *Precipitation test:* This is a quantitative modification of Dorfman's qualitative test for mucopolysaccharides(8) and is based on the fact that these highly charged macromolecules combine, at pH 3.7, with bovine serum albumin to form an insoluble complex which exists as a turbid solution for about 20-30 minutes before precipitation. A standard curve is prepared using chondroitin sulfate-A as the known mucopolysaccharide, and turbidity is measured at 600 mμ. Linear relationships were obtained at values ranging from 0-60 mg of chondroitin sulfate-A/liter. The turbidities observed with other acid mucopolysaccharides, while different, were of the same order of magnitude.

2. *Reducing test:* Mucopolysaccharides, like most other polysaccharides, have little reducing power. Upon acid hydrolysis the glycosidic bonds break, freeing most of the reducing anomeric carbons. When dialyzed urine is initially analyzed for reducing power and then acid hydrolyzed and tested again, the increase in reducing groups is proportional to the amount of mucopolysaccharide present. Large quantities of glycoproteins and mucoproteins would be the only interfering substances to be found in dialyzed urine. Since it is the change in reducing power which is being measured, large initial values such as would be obtained with the urine of diabetics will not yield false positives.

The optimum time and HCl concentration for polysaccharide hyrdolysis were determined to be 0.75 *N* HCl and 60 minutes. The Folin-Wu method(9) was used to measure reducing power.

3. *Periodate oxidation test:* Adjacent hydroxyl groups or adjacent hydroxyl and amino groups in organic compounds are quantitatively oxidized by periodate at pH 4.0. Mucopolysaccharides contain many such groups and are readily oxidized by periodate. Once again, only glycoproteins and mucoproteins, in great quantity, could interfere. The assay method used is a potentiometric titration of the iodine liberated from a KI solution by the excess periodic acid left in a mucopolysaccharide-periodic acid reaction mixture(10).

The sensitivity of this test may be approximately doubled by preliminary hydrolysis of the urine as described for the reducing test.

Results. The results of the application of these 3 tests to the urines of members of the affected family are shown in Table I.

TABLE I. Quantitative Estimation of Urinary Mucopolysaccharides.

	Genealogy No.	Precipitation[1] Test	Reducing[2] Power	Periodate[3] Uptake	Periodate[3,4] Uptake
Gargoyles	41	122	81	7.0	9.0
	45	96	130	9.4	15.6
	51	33.5	28.2	3.4	3.6
Known Carriers	9	6.8	17.2	3.6	5.4
	20	1.8	2.5	2.6	
	31	4.8	3.8	3.7	
	32	6.2	7.5	2.6	
	33	3.7	.9	2.5	
Sib of Gargoyles					
Male	46	9.7	34.7	3.3	6.7
Female	42	8.7	1.8		
	43	13.0	13.5	3.6	
	44	9.8	17.2	3.7	
	47	10.8	15.9	3.5	
	49	6.2	1.2	3.2	
	50	6.5	6.2		
	52	10.8	7.7	3.1	

[1] Expressed as mg/L chondroitin sulfate-A.
[2] " " mg/L glucose.
[3] Milliequivalents periodate/L.
[4] Following acid hydrolysis.

TABLE II. Relationship of Urinary Mucopolysaccharides to Creatinine Excretion

Genealogy no.	Specific gravity	Creatinine mg/ml	Precipitation test mg/L	Uronic A. mg/L	Precipitation test: creatinine ratio	Uronic A.:creatinine ratio
20 (C)	1.012	.60	3	10	5	17
31 (C)	1.013	.80	8	15	10	19
	1.027	.82	10	—	12	—
32 (C)	1.013	.73	8	22	11	30
	1.012	.30	7	8	23	27
33 (C)	1.018	1.77	22	24	12	14
	1.013	.52	4	12	8	23
41 (G)	1.013	.40	166	75	415	188
	1.021	.76	112	86	147	133
42	1.022	.67	18	20	27	30
43	1.026	1.20	19	30	16	25
44	1.021	1.27	28	31	22	24
45 (G)	1.023	.66	—	69	—	105
	1.013	.33	84	92	255	280
46	1.024	.90	17	40	19	44
	1.023	.77	7	19	10	25
47	1.008	.47	4	13	8	28
49	1.021	.97	12	22	12	23
50	1.024	1.17	10	27	9	23
51 (G)	1.007	.17	62	49	365	288
	1.014	.32	60	40	188	125
52	1.008	.27	5	11	18	41

The precipitation test proved to be the most rapid method. It is clear from the table that clinically proven gargoyles excrete at least 3 times as much mucopolysaccharide as do other individuals. From a survey of a large number of normal individuals, it was ascertained that an arbitrary value of 7-9 mg chondroitin sulfate-A per liter could be set at the upper limit of normal. Usually, normal values ranged from 1-4 mg per liter.

The change in reducing power was perhaps the most sensitive of the tests with an arbitrary value equivalent to 20 mg per liter of glucose established as the upper limit of normal in a survey of a large number of non-gargoyle urines.

The periodate test is of considerably less value. Even after hydrolysis which doubled the periodate uptake, the differences between normal individuals and gargoyles were at most 1½-to 2-fold. Due to the high oxidation potential of the periodate, other substances in the urine may also be oxidized, although dialysis should have decreased such interference.

The data given represent only single determinations. Parallel determinations on the same urine samples yielded the same figures. When more than one urine sample from any one individual was tested the values, while they showed considerable variation, still fell within the characteristic range for affected or normal individuals.

A further series of studies was carried out using large pooled samples of urine from some of the individuals in the genealogy. As a means of correlating the mucopolysaccharide excretion with an independent measure of metabolic activity, the relationship of urinary mucopolysaccharide excretion to creatinine excretion was measured using the precipitation test and the uronic acid assay of Dische(11) and the creatine analysis of Bonsnes and Taussky(12). The results are shown in Table II. In the cases of known gargoyles, results of analyses of different samples are shown as examples of the variations found.

In general, the various methods for determining urinary mucopolysaccharide levels yielded parallel data which indicate that clinically affected gargoyles excrete significantly increased quantities of mucopolysaccharide. The known carriers and most of the siblings are in or near the normal range. Based on these quantitative data it is not yet possible to identify carriers by these procedures.

Since the analytical methods employed yield values for total mucopolysaccharide without distinguishing among the various types which may be present, the present results do not preclude the existence of qualitative differences between carriers and normal individuals which would not be quantitatively evidenced. Such qualitative differences are currently being investigated.

Summary. The urinary mucopolysaccharide excretion of a family with the sex-linked form of gargoylism has been quantitatively studied, using several methods. The results indicate that neither carriers nor unaffected siblings excrete significantly higher than normal quantities of total acid mucopolysaccharides.

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Hexosamine and Hydroxyproline Alterations in Chronically Sun-Damaged Skin.* (26988)

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The wrinkling, loss of elasticity, and other changes in human skin associated with aging are markedly accentuated by chronic sun damage. Unexposed skin of elderly persons does not show these alterations to the same degree as exposed areas and may look decades younger than uncovered skin(10). Histochemically, the dermis of sun-damaged skin (actinic elastosis) displays increased staining for acid mucopolysaccharides (*e.g.* Alcian blue, Mowry colloidal iron) and elastin-like material (*e.g.* orcein, Verhoeff-VanGieson) (6,9). The relationship to true elastin and collagen of the latter fraction is still uncertain(7). This study was undertaken to quantitate hexosamine and hydroxyproline in sun-damaged skin as compared to unexposed skin, and to localize the abnormality.

Materials and methods. Chronically sun-damaged skin of the forearms of 3 volunteers was removed with a Brown dermatome under local anesthesia; elliptical areas of similarly damaged skin were excised from the forearms or neck of 6 other long-time residents of central Florida.† Control skin was removed at autopsy from the Y incision of the abdomen of 12 adults whose deaths resulted from a variety of causes. All skin was frozen until

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† Skin from human volunteers was obtained through the cooperation of Dr. Charles C. Tindall, Kissimmee, Fla.

TABLE I. Hexosamine and Hydroxyproline of Chronically Severely Sun-Damaged and Control Skin

	Chronic sun-damaged dermis	Control dermis
No.	9	12
Age	67.8 ± 9.3*	53.5 ± 12.5
Hexosamine (μm/100 mg dry wt)	3.4 ± .9	2.0 ± .5
Hydroxyproline (μm/100 mg dry wt)	35.0 ± 11.1	66.0 ± 3.6
Hexosamine/hydroxyproline ×'s 10	10.7 ± 4.3	3.0 ± .9

* Mean and standard deviation.

ready for use. Small pieces of the skin from all groups were fixed for 24-48 hours in 10% formalin for histological and histochemical study. Skin removed with the Brown dermatome was between 0.3 to 0.38 mm in thickness. The upper 0.3 mm of the other skin specimens was removed using the Castroveijo keratotome(2).

A similar 0.3 mm section of skin from the lower-most dermis of 5 specimens of excised sun-damaged defatted skin was also removed with the keratotome to compare with the upper section of dermis from the same individual, since histologic changes are demonstrable only in the upper ⅓ to ½ of sun-damaged dermis(9). The epidermis was heat separated from the dermis using a modification of the Baumberger method(1). The dry weight of dermis was obtained by desiccation in a vacuum oven and the dermis hydrolysed with 4 N hydrochloric acid for 15 hours at 100°C in sealed tubes. Neutralized aliquots of the hydrolysate were taken for hydroxyproline(8) and hexosamine(4) determinations. Paper chromatography was used to confirm the identity of the amino sugars.

Results. All sections of sun-damaged skin showed increased staining in the upper dermis with the Mowry colloidal iron and the orcein stains as compared to the control material. Hexosamine in the sun-damaged skin was significantly increased over that in the control skin, whereas the hydroxyproline content was significantly lower than in the controls (Table I). The hexosamine/hydroxyproline ratio of the controls was lower than that of sun-damaged skin.

The comparison of the upper and lower-most dermis of 5 individuals is shown in Table II. Differences between hexosamine, hydroxyproline, and hexosamine/hydroxyproline ratios are statistically significant.

Paper chromatography of the amino sugars indicated that glucosamine exceeded galactosamine in both sun-damaged and control skin samples.

Discussion. The hexosamine and hydroxyproline alterations of elastotic skin represent a reversal of the pattern found in unexposed skin with age(11). The decrease in hydroxyproline accompanying epidermal carcinoma induction by topical application of the carcinogen methylcholanthrene(5) is interesting in view of the similar finding in sun-damaged skin whose susceptibility to development of malignancy is well-documented(3). The re-

TABLE II. Hexosamine and Hydroxyproline in Upper and Lower Dermis of 5 Individuals with Chronically Severely Sun-Damaged Skin

	Upper dermis	Lower dermis
Hexosamine (μm/100 mg dry wt)	3.6 ± 1.1*	1.6 ± .14
Hydroxyproline (μm/100 mg dry wt)	41.1 ± 6.8	58.1 ± 9.0
Hexosamine/hydroxyproline ×'s 10	8.9 ± 3.1	2.8 ± .5

* Mean and standard deviation.

duced level of hydroxyproline does not, however, define the identity of the elastin-staining material, since either an increase of true elastin or a degradation of collagen could result in the lower value.

The excess of glucosamine over galactosamine combined with the hyaluronidase lability of the acid mucopolysaccharide staining in sun-damaged skin(9) suggest hyaluronic acid is the major mucopolysaccharide in sun-damaged skin.

Summary. 1. The upper dermis of chronically sun-damaged human skin contains more hexosamine and less hydroxyproline than the upper dermis from unexposed areas. 2. The increased hexosamine confirms the presence of increased acid mucopolysaccharides seen histologically in sun-damaged skin, but the decreased hydroxyproline does not identify the elastin staining material as either elastin or "degraded" collagen. 3. The upper dermis of sun-damaged skin has higher hexosamine and lower hydroxyproline levels than the lower-most dermis of the same specimen, thus the localization of the biochemical alteration corresponds with the localization of the histologic abnormality.

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Is There a Heme-Stimulating Factor in Human Plasma?* (26989)

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Many recent observations have strengthened the original studies of Carnot and Deflandre(1) in experimental animals, suggesting a humoral mechanism controlling erythrocyte production. Plasmas from patients with various hematological disorders have also been found to stimulate erythropoiesis in experimental animals(2). Linman *et al*(3) have suggested that there are probably 2 factors in plasma which exert control over erythropoiesis; one, a thermostable factor which stimulates division of erythroblasts and another factor which is only relatively thermostable, which enhances hemoglobin synthesis.

Various attempts have been made to demonstrate the hemoglobin stimulating factor *in vitro*. Normal rabbit serum has been shown to have a marked stimulating effect on rate of incorporation of C^{14}-labelled glycine into heme by rabbit bone marrow(4). This heme-stimulating factor in serum was thermolabile. There are however conflicting reports regarding the effect of plasma on hemoglobin formation as measured by uptake of radioiron into heme. Using an hemolysate of chicken red cells as the enzymic source and glycine as substrate, Goldberg *et al*(5) failed to show any difference in uptake of radioiron into heme between deproteinized plasmas of normal chickens and of chickens made anemic by either bleeding or phenylhydrazine. Uptake of radioiron into heme was much greater when saline was substituted for any of these plasma extracts. In these experiments the iron content of each protein-free extract was determined and sufficient iron had been added to keep constant the total iron content of every extract. Nakao *et al*(6), using an hemolysate of chicken red cells as the enzymic source and δ amino levulic acid as substrate, observed that addition of plasmas from patients with polycythemia, acute myeloid leukemia and aplastic anemia produced an increased incorporation of radioiron into heme when compared to normal human plasma. The plasma from the patient with aplastic anemia apparently stimulated uptake of radioiron into heme a hundred-fold. They consequently postulated that the plasma in these states had heme-stimulating activity. It should be noted that in the latter experiments a protein-free extract was not used except for 2 plasmas in which no heme-stimulating activity was obtained. Although the above authors measured the iron content of each plasma sample, they did not measure the residual iron-binding capacity.

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The discrepancy between the results of Goldberg *et al*(5) and Nakao *et al*(6) is of interest since the method of measuring heme formation was similar. The following study was undertaken to determine the cause of these differences. Two obvious factors were the varying size of the iron pools and the varying iron-binding capacities of the plasmas used in the studies of Nakao *et al*(6). The effect of these factors on iron-incorporation into heme was assessed by (1) addition of human plasmas of known residual iron binding capacity, obtained in a variety of hematological disorders and (2) by addition of increasing amounts of iron to a standard aliquot of plasma which thus modified the residual iron-binding capacity as well as the total iron pool of the plasma.

Method. Five ml of chicken red cell hemolysate prepared by the method of Dresel and Falk(7), δ amino levulic acid (4.1×10^{-4}M) and 1 μc radioiron ($Fe^{59}Cl_3$) (specific activity about 2 μc/μg) were incubated with 2 ml of human plasma separated from heparinised blood. The pH was maintained at 7.4 by addition of 0.45 M Tris buffer (2-amino-2-hydroxymethylpropane 1:3 diol). The mixture was incubated with constant shaking at 37°C for 3 hours and the reaction stopped by addition of 1 ml M sodium cyanide. Carrier hemoglobin prepared by the method of Goldberg *et al*(5) was then added to facilitate the isolation of heme. The hemolysate was homogenised in a Waring blender for 3 minutes and centrifuged at 1600 g for 20 minutes to remove tissue debris. The heme was then isolated from the supernatant fluid by the method of Fischer(8) and purified and recrystallised by the method of Shemin *et al*(9). Radioactivity of weighed samples of the isolated heme was determined in a well-type gamma ray scintillation counter with a counting efficiency for iron of 22%. The total number of counts present in the heme was obtained by multiplying the counts per milligram of heme by the total number of milligrams of heme present in the original hemolysate. Results are expressed as percentage of the added radioiron which was incorporated into heme. There is some variation in the iron-incorporating activity of different chicken hemolysates(5). For this reason 2 ml saline was substituted in every experiment for 2 ml of plasma in control flasks and the iron-incorporation into heme with each plasma sample was related to these saline controls in the following way:-

$$\begin{array}{c}\text{Calculated}\\ \text{iron-incorporation}\\ \text{with plasme sample}\end{array} = \begin{array}{c}\text{Actual}\\ \text{iron-incorporation}\\ \text{with plasma}\end{array} \times \frac{\begin{array}{c}\text{Maximum iron-incorporation}\\ \text{of all saline controls}\end{array}}{\begin{array}{c}\text{Iron-incorporation with}\\ \text{respective saline control.}\end{array}}$$

Serum iron concentration and total iron-binding capacity of plasma were measured by the method described by Ramsay(10).

Results. Comparison of effects of different plasmas. The iron-incorporation into heme was measured when 2 ml samples of the plasmas of 16 patients were added to the system (Table 1). The plasmas were obtained from 5 hematologically normal subjects (Cases 4, 8, 9, 10, 11), 2 patients with a low plasma iron level (Cases 6, 7), one with hemochromatosis (Case 16), one with an un-

TABLE I. Effect of Plasmas of Varying Residual Iron-binding Capacities on Percentage Uptake of Radioiron into Heme.

Case no.	Diagnosis	Hemoglobin (G %)	Serum iron (μg%)	Total iron-binding capacity (μg%)	Residual iron-binding capacity (μg%)	Calculated % uptake of Fe^{59}
1.	Lead Poisoning	9.9	65	489	424	.28
2.	Secondary Polycythemia	16.4	10	336	326	.03
3.	*Idem.*	18.4	60	378	318	.02
4.	Myocardial Infarction	15	72	384	312	.26
5.	Idiopathic acquired hemolytic anemia	10.6	70	308	238	.04
6.	Mild Staphylococcal Infection	14.8	36	270	234	.07
7.	Essential Hypertension	15.7	20	254	234	.31
8.	Hematologically normal	14.8	98	326	228	.10
9.	Myocardial Infarction	13.9	78	300	222	.03
10.	Duodenal Ulcer	14.2	76	274	198	.08
11.	Cerebral Ischemia	15.7	60	222	162	.57
12.	Hypoplastic Anemia	8.3	130	288	158	.62
13.	Monocytic Leukemia	8.4	157	237	80	.045
14.	Aplastic Anemia	5.9	208	276	68	3.15
15.	Myocardial Ischemia	15.4	176	232	56	1.25
16.	Hemochromatosis	14.8	160	198	38	2.08

MAXIMUM UPTAKE OF RADIOIRON OF ALL SALINE CONTROLS = 12%

explained high serum iron (Case 15), and 7 with various disorders involving the hemopoietic system (Cases 1, 2, 3, 5, 12, 13, 14). There was a marked increase of iron-incorporation with addition of the plasmas from 3 patients in each of whom the level of plasma iron was markedly raised and the residual iron binding capacity correspondingly reduced. (Table 1, Cases 14, 15, 16).

Effect of addition of varying amounts of iron to normal plasma. Increasing amounts of iron (4-8 μg,) as ferric ammonium sulfate were added to 2 ml aliquots of a normal plasma so that there was a progressive increase in saturation of the iron-binding capacity(11). There was a sharp increase in iron-incorporation into heme as the iron-binding capacity approached saturation (Fig. 1). The addition of iron in excess of total iron-binding capacity of plasma did not, in the iron concentrations used, alter the percentage

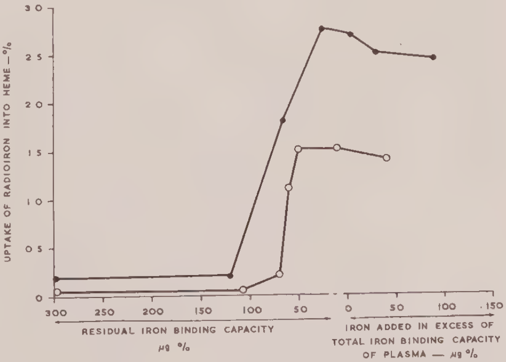

FIG. 1. Effect on heme synthesis of normal plasma to which varying amounts of iron have been added.

uptake of radioiron into heme as compared with saturated plasma. Under all circumstances the percentage uptake of radioiron into heme was less in the presence of plasma than in the presence of an equal volume of saline.

Discussion. There is ample evidence for the presence in human plasma of a humoral factor stimulating erythropoiesis. The evidence for a heme-stimulating factor is still unconvincing and every report relating to this should be critically appraised. Nakao *et al*(6) claimed that plasmas from a variety of hematological disorders had heme-stimulating activity. We have used an identical technique to assess this activity in the plasmas of 16 subjects but have found that increased heme-formation occurred with only 3 of these plasmas, in each of which the residual iron-binding capacity was greatly diminished, 68 μg per cent or less. In contrast to Nakao's results the plasmas from patients with secondary polycythemia (cases 2 and 3) and hypoplastic anemia (case 12) showed no evidence of increased heme formation but each of these patients had a residual iron-binding capacity greater than 158 μg per cent. In the plasma of one patient with monocytic leukemia (case 13) in which the residual iron binding capacity was 80 μg per cent, no increase in heme formation occurred.

These results suggested the importance of the residual iron-binding capacity of the plasma samples in a system using radioiron as a tracer for measuring heme synthesis. The so-called heme-stimulating activity of the plasmas observed by Nakao *et al.* could be explained by variability in residual iron-binding capacity. This suggestion is corroborated by the results noted in Fig. 1. An increased iron-incorporation into heme occurred only on addition of plasmas in which the residual iron-binding capacity had been partially or completely saturated by non-radioactive iron. In these experiments one μc radioiron (containing 0.6 μg iron) was added to each flask. Thus a 2 ml sample of plasma with a residual iron-binding capacity of 30 μg per cent could render this quantity of radioiron unavailable for heme synthesis. Lochhead and Goldberg(12), have already shown that radioiron, when added to plasma, is bound to siderophilin and is not readily released for heme synthesis unless certain reducing substances are added. The differences noted in the plasma samples with a residual iron-binding capacity greater than 30 μg per cent may well be accounted for by a varying level of physiological reducing substances present in each tissue system. These considerations have led us to conclude that the apparent heme-stimulating activity of the human plasmas noted by Nakao *et al.*(6) can be explained by the varying residual iron-binding capacity of these plasmas.

Summary. (1) Nakao *et al.*(6) have suggested that plasma samples from a variety of hematological disorders possess heme-stimulating activity as compared to normal plasma. This activity was determined by an *in vitro* technique in which radioiron was used to measure heme synthesis. (2) The present studies have shown that any marked increase in heme formation, determined by the same technique, could be explained by diminution of the residual iron-binding capacity of the plasma sample. (3) In further experiments the residual iron-binding capacity of normal human plasma was modified by addition of varying amounts of non-radioactive iron. Heme formation in the presence of the plasma was directly related to degree of saturation of the residual iron-binding capacity. (4) It is concluded that no definite evidence has yet been obtained for the presence of a specific heme-stimulating factor in human plasma.

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Renal Localization and Persistence of Type 12 Streptococcal M-Protein.* (26990)

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The hypothesis that acute glomerulonephritis results from a hypersensitivity reaction has prompted investigations to explain why such reactions primarily involve the kidney. With respect to the possibility that the antigen might be a foreign protein, the association between Group A streptococcal infection, especially of certain types, *e.g.* Type 12, and the subsequent development of glomerulonephritis has focused attention on streptococcal substances.

Schmidt(1,2) reported that the Group A carbohydrate could be detected, using immunofluorescent techniques, in renal tubular cells of mice for up to 30 minutes following intravenous injection, and that a streptococcal protein could be demonstrated in cells of the reticulo-endothelial cells (RES) for as long as 60 hours. Kaplan(3) has extended the observations on the distribution of streptococcal proteins. He found that when small amounts of acid extracted M-protein were injected into mice, localization occurred largely in the phagocytic cells of the RES and within glomeruli. The M-protein was found to persist in glomeruli for at least 8 days, whereas outside the kidney virtually none was demonstrable by the fourth day.

On the basis of these observations, it might be postulated that the initial event in the pathogenesis of acute glomerulonephritis involves the deposition of M-protein within glomeruli and that subsequently, as circulating antibody against M-protein is produced, this antibody combines with the protein persisting in glomeruli, thereby damaging these structures. However, Kaplan detected no differences in the glomerular localization or persistence of M-protein from Types 1, 5, 12 and 19 Group A streptococci, so that even if the mechanism postulated above is true, the nephritogenic action of certain streptococcal types could not be explained simply on the basis of certain M-proteins having the special property of localizing and persisting in glomeruli.

Furthermore, the possibility exists that the distribution of M-protein observed by Kaplan reflects alterations in the protein produced during the process of its isolation. Thorbecke, Maurer and Benacerraf(4) showed that certain acid treated serum proteins may be rapidly cleared from the circulation by the kidney, whereas the unmodified protein is not.

The present investigation was undertaken to determine whether M-protein isolated with the use of a phage lysin would exhibit the same *in vivo* distribution as the material prepared by the relatively drastic procedure of hot acid extraction. The behavior of both preparations of M-protein was contrasted with that of acid denatured bovine serum albumin (BSA). It was found that phage prepared M-protein did localize and persist in glomeruli of mice, where it was demonstrated for as long as 16 days after a small intravenous injection.

Materials and methods. Animals: Swiss-Webster albino mice of both sexes, weighing 25-30 g were used.

Streptococci: Nass strain, Group A Type 12 streptococci, isolated during a focal outbreak of glomerulonephritis was generously

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provided by Dr. C. H. Rammelkamp, Jr. (Cleveland Metropolitan General Hosp., Cleveland, Ohio). The M-protein production of this strain was enhanced by repeated intraperitoneal passage in mice. Purity was frequently checked by plating, Gram staining, grouping, and typing. Strains of Types 1 and 3 were obtained from Dr. A. Bernheimer (New York Univ. School of Med.)

Acid denatured BSA was produced according to Maurer(5).

Antisera: Antisera specific for Type 12 were supplied by Dr. C. H. Rammelkamp, Jr. Antisera against other types were obtained from the U.S. Public Health Service Communicable Diseases Laboratory, Atlanta, Ga. Anti-whole streptococcal serum was prepared by repeated injection of washed heat and formalin killed bacteria into rabbits. The streptococci were grown in trypticase soy broth (Baltimore Biol. Lab., Baltimore, Md.). Antisera against acid denatured BSA were produced in rabbits(5). Sheep anti-rabbit gamma globulin antiserum was obtained through the courtesy of Dr. G. J. Thorbecke (New York Univ. School of Med.)

Fluorescein-conjugated antibody: Sheep anti-rabbit gamma globulin was conjugated with fluorescein using the isothiocyanate compound according to the method of Riggs *et al.*(6,7). Non-specific staining was completely eliminated by appropriate elution from a DEAE cellulose column(8) and by absorption with mouse tissue homogenates(3).

Fluorescent antibody techniques: Mice were killed by decapitation and pieces of lung, liver, spleen, and kidney were frozen by immersion in liquid nitrogen and stored at —22°C. until sectioned in a cryostat. Following sectioning, the tissue was fixed for 20 minutes in acetone. Sections were stained by the indirect technique, except for those experiments in which rabbit gamma globulin was looked for and in this case the direct technique using fluorescein-conjugated sheep anti-rabbit gamma globulin was employed. The following observations were considered to demonstrate the specificity of positive staining: 1) failure to stain normal mouse tissues; 2) failure to stain smears of Types 1 or 3 streptococci; 3) failure to stain tissues of mice injected with Type 12 M-protein using antisera directed against Types 1, 3, 19, or 25, or Group A polysaccharide; and 4) failure to stain tissues of experimental animals with Type 12 antiserum which had been absorbed with Type 12 M-protein(3). Tissues were also fixed in buffered formalin and hematoxylin and eosin stained sections were prepared.

M-protein: M-protein was prepared by 2 methods. Acid extracted M-protein, Type 12, was prepared from washed bacterial cell walls(9) according to the method described by Lancefield and Perlmann(10). M-protein was also prepared from cell walls by the use of a lysin associated with streptococcal bacteriophage(11). This work was done in collaboration with Dr. Richard M. Krause (Rockefeller Inst. New York). In both cases M-protein was precipitated by 0.33-0.6 saturated ammonium sulfate. All fractions were tested for precipitin reactions by double diffusion in agar(12) and immunolelectrophoresis(13) against typing, grouping, and anti-whole streptococcal antisera. Both preparations showed strong single lines against the anti-Type 12 M sera but not against the other typing sera. Untreated preparations of both materials gave no reaction with antiserum against the Group A polysaccharide, but when the lysin prepared M-protein was first treated with *Streptomyces albus* enzyme, a proteolysin, a faint line was evident(11). With both acid and phage prepared M-protein, the anti-whole streptococcal serum gave a very strong single line, corresponding to M and several very much fainter lines. Protein content was determined by Biuret and microKjeldahl methods.

Experimental. Mice were divided into 7 groups as follows:

Group I consisted of normal untreated animals.

Group II consisted of 8 mice given 0.5 mg of acid extracted M-protein and 25 mice given 1.0 mg of acid-extracted M-protein. Material in this group, and in those described

TABLE I. the Persistence of Group A Type 12 Streptococcal M-Protein and Acid Denatured BSA in Glomeruli of Mice After I.V. Injection.

	Material injected					
	Acid extracted M		Phage Lysin prepared M		Denatured BSA	
	Dose (mg)		Dose (mg)		Dose (mg)	
Day killed post-injection	.5	1.0	.5	1.0	.5	1.0
1	1/1*	1/1	1/1		1/1	
2	1/1		1/1		1/1	
3	1/1		1/1		1/1	2/2
4	1/1	2/3	1/1	3/3	0/1	1/2
5	1/1	3/3	1/1	2/2	0/1	
6	1/1		1/1		0/1	
7	1/1		0/1		0/1	
8	1/1	2/2	1/1	2/2	0/1	0/2
9		1/2		2/2		
12		3/4	1/3	2/3		
16		2/3		2/3		
20		0/4		1/3		
25		0/3		0/3		

* Indicates number of animals with glomeruli containing detectable amounts of M-protein or acid denatured BSA over number of mice studied.

below was administered intravenously in the tail vein in a volume of 0.5 ml.

Group III consisted of 11 mice given 0.5 mg and 21 mice given 1.0 mg of phage lysin prepared M-protein.

Group IV consisted of 8 mice given 0.5 mg and 6 mice given 1.0 mg of acid denatured BSA.

All of the animals in the above groups were killed on various days following injection according to the schedule shown in Table I.

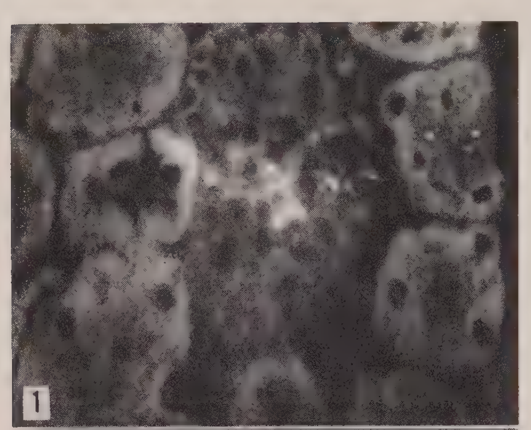
Groups V, VI and VII consisted of 6 mice each. Animals in these 3 groups were given 1.0 mg of acid extracted M-protein, phage lysin prepared M-protein and acid denatured BSA respectively. One day later the animals in each group were given an intravenous injection of 0.5 mg of the appropriate antibody protein intravenously. Three of the mice in each group were killed 2 days later and the remaining mice 3 days later.

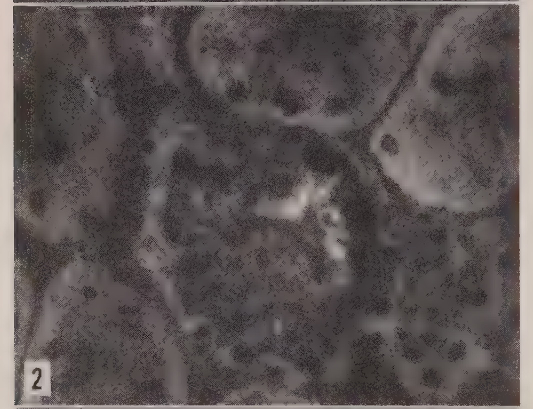
Organs from all animals were examined by fluorescent and light microscopy.

All of the animals given either acid extracted or phage lysin prepared M-protein alone showed distribution and persistence patterns essentially the same as those described by Kaplan(3). Fluorescent material was present in the phagocytic cells of the liver, spleen, and to a lesser extent, of the lungs for the first 2 days following injection. M-protein was not apparent in any other location examined, outside of the kidney, including hepatic parenchymal cells, alveolar walls or pulmonary capillaries, or in the germinal centers of the spleen. By the third day only traces could be detected in liver or spleen and none was apparent in the lung. At 4 days and later no fluorescent material was demonstrated except in glomeruli. There appeared to be no difference between animals given 0.5 or 1.0 mg.

The picture in the kidneys was quite different. Throughout this period M-protein was evident in high concentration in almost all glomeruli following administration of either preparation. Only rare deposits were noted in the interstitial tissues. The only difference observed between the 2 materials occurred during the first 2 or 3 days following administration, when the phage lysin prepared material showed a moderate amount of tubular deposition, fluorescence being present in nuclei, cytoplasm and tubular lumens. This staining was not seen after the fourth day.

Staining in individual glomeruli characteristically consisted of small aggregates of fluorescent material that at times assumed a globular configuration. While the M-protein appeared to be deposited intracellularly it was impossible to determine if capillary endothelial cells or epithelial cells were principally





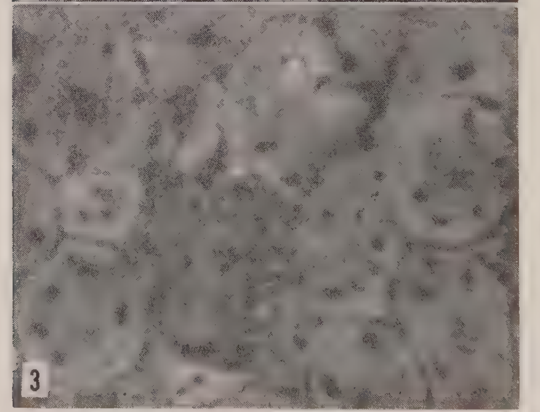


FIG. 1. Type 12 M-protein (phage lysin prepared) in mouse glomerulus 4 days after injection of 1.0 mg I.V. Stained with rabbit anti-Type 12 M-protein antisera followed by fluorescein conjugated sheep anti-rabbit gamma globulin antibody (528 ×).

FIG. 2. Phage lysin prepared Type 12 streptococcal M-protein in mouse glomerulus 16 days after injection of 1.0 mg I.V. Stained as in Fig. 1 (528 ×).

FIG. 3. Normal mouse glomerulus (528 ×).

involved. Nuclei of involved cells did not stain (Fig. 1).

During the first 4 days the amount of M-protein, using either acid extracted or phage lysin prepared material, in the kidney seemed to decrease as evidenced by a diminution in the extent of staining within individual glomeruli. Throughout this period M-protein was readily detectable in the urine by means of capillary precipitin tests. From the fourth to the eighth day after administration there appeared to be little change and the great majority of glomeruli continued to show deposits. Between days 8 and 12 the number of involved glomeruli decreased considerably but fluorescence could still be seen in many glomeruli at the end of this time. By the sixteenth day only occasional glomeruli showed M-protein (Fig. 2). In these the fluorescence was quite bright although not extensive. With one exception no positive results were obtained beyond 16 days.

Of the animals given M-protein alone, only 3 showed evidence of pathologic changes in the form of equivocal focal inflammation in glomeruli. Two of these mice had been given 1.0 mg of phage lysin prepared material and one animal had been given 1.0 mg of acid extracted M-protein; all were killed 16 days after injection.

In Group IV, in which animals were given acid denatured BSA, during the first 2 or 3 days only small amounts of acid denatured BSA could be detected in cells of the RES, glomeruli or renal tubular cells. Large amounts of the modified BSA were readily detectable in the urine as determined by precipitin reactions with specific antibody. By the fourth day no fluorescence was observed in any of the tissues of any animal in this group. No pathologic changes were noted.

No lesions appeared in the kidneys or other organs of those animals given antibody after receiving either M-protein or denatured BSA. Fluorescent microscopy revealed only very small amounts of rabbit gamma globulin, presumably specific antibody, in the glomeruli. Animals which received the acid denatured BSA and antibody directed against it showed considerably less renal deposition of the antibody than animals given the M-protein system.

Discussion. The relationship between

streptococcal infection and acute hemorrhagic glomerulonephritis in man has led to a consideration of various streptococcal substances as either participants in an immune reaction within glomeruli or as substances capable of directly damaging glomeruli(14).

The association between nephritis and certain types of Group A streptococci warrants consideration of the possibility that the type specific M-protein plays a role in the pathogenesis of the renal damage. It appears, in view our findings, that M-protein possesses the property of localizing and persisting in the glomeruli of mice for many days. This property does not seem to be the result of alterations in the protein occurring during its isolation, since there was no apparent difference in the behavior of either M-protein preparation.

The ability of M-protein to localize and persist in glomeruli for long periods of time renders plausible the hypothesis that this streptococcal antigen participates in pathogenesis of acute nephritis on an immune basis. Such a period of time is more than the minimal limit required for production of antibody, and that this material can be detected by immunofluorescent methods indicates that it has retained its capacity to combine with antibody during this interval. The demonstration that circulating antibody, even in small amounts, has access to the antigen for at least 4 days after its administration further emphasizes this point. The failure to produce glomerular lesions by administration of antibody after injection of M-protein under the conditions employed here does not support this hypothesis, but different schedules or dosages might lead to the production of lesions.

While only equivocal glomerular abnormalities were observed in a small number of the animals injected with M-protein alone it is possible that administration of larger amounts, greater than 1 mg, might produce lesions. In this connection the possibility should be noted that M-protein might possess certain tissue damaging properties, especially in the case of the lysin preparation. Cromartie and his associates(15) have observed that a polysaccharide-protein complex obtained from Group A streptococci is toxic for rabbit connective tissue. Krause has provided evidence for a similarity between this substance and phage lysin prepared M-protein by demonstrating that the latter is associated with a polysaccharide moiety(11).

Summary. The findings of Kaplan with regard to the renal localization of Type 12 streptococcal M-protein have been confirmed and extended. It has been shown that this property is not the result of denaturation during preparation. The significance of these findings in regard to hypotheses concerning the pathogenesis of acute nephritis is briefly discussed.

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Lipid Distribution in Lipoproteins Separated by Polyanion Precipitation. (26991)

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The isolation of human β-lipoproteins by polyanion precipitation methods has been the subject of several recent reports(1,2,3). Since the technique offers the advantages of speed and simplicity, and does not require specialized equipment, it has considerable potential as a research tool.

The completeness of separation of β-lipoproteins by polyanion precipitation methods has been evaluated by paper electrophoresis (4) with human sera and by ultracentrifugation with both human and animal sera(5).

Since the chick is widely employed as an experimental animal for study of lipid metabolism, data are presented concerning the effectiveness of polyanion precipitation methods in this species. This report also presents data on the lipid content of plasma and of the supernatant fraction (α-lipoprotein) following polyanion precipitation for the chick, and for other animal species.

Methods. Blood samples were obtained from experimental animals by cardiac puncture. Unless otherwise noted, the animals were fed a stock diet. Human blood samples were obtained from volunteers by venipuncture.

The polyanion precipitation method described by Florsheim and Gonzales(5) was employed. This procedure uses Mepesulfate, the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside (Hoffmann-LaRoche), as the polyanion. The completeness of β-lipoprotein precipitation was evaluated by paper electrophoresis and by ultracentrifugation. Electrophoretic separations were made of the plasma and the supernatant after Mepesulfate precipitation; a Durrum-type cell was employed with a barbiturate buffer (pH 8.6; ionic strength 0.075). The strips were stained with oil red O to locate the lipoprotein bands. The plasma and supernatant obtained after Mepesulfate precipitation were subjected to ultracentrifugation by the method of Havel *et al.*(6) after initial adjustment to a density of 1.063 with sodium chloride. Completeness of separation was estimated by the cholesterol content of the α-($D>1.063$) and β-($D<1.063$) lipoprotein fractions.

Lipid distribution in the α- and β-lipoproteins was estimated by determining lipid content of the plasma and the supernatant resulting from Mepesulfate precipitation, values for β-lipoprotein lipid being obtained by difference. Lipids were extracted with chloroform:methanol (2:1). In some cases, the extracted lipids were fractionated by silicic acid chromatography by a modification of the method of Hirsch and Ahrens(7) previously described(8). Cholesterol and lipid phosphorus were determined by the method of Searcy and Bergquist(9) and Fiske and Subbarow(10), respectively. Glycerides were determined by a modification of the method of Van Handel and Zilversmit(11) previously described(12).

Results and discussion. Chick plasma was subjected to paper electrophoretic analysis before and after Mepesulfate precipitation. Mepesulfate precipitation resulted in disappearance of the β-lipoprotein band. Total cholesterol determinations demonstrated that 70-80% of the total plasma level of cholesterol was present in the supernatant after Mepesulfate precipitation (α-lipoprotein fraction). This finding is in contrast to values reported for the chicken. Orma(13) reported 50-70% of the total cholesterol to be in the β-fraction as separated by paper electrophoresis.

To verify the results obtained, cholesterol was determined in high ($D>1.063$) and low ($D<1.063$) density lipoproteins separated by ultracentrifugation. The level of total cholesterol in the high density fraction of plasma and the supernatant fraction obtained after Mepesulfate precipitation were identical (104 vs. 105 mg/100 ml). The amount of cholesterol remaining in the low density lipo-

TABLE I. Plasma and α-Lipoprotein Cholesterol in Chicks Fed Different Diets.

Dietary treatment	No. of samples	Plasma cholesterol, mg/100 ml Total	α-Lipoprotein fraction
20% protein	8	102 ± 7*	73 ± 7*
20% " + 2% cholesterol	7	523 ± 51	94 ± 10
18% " (stock diet)	23	147 ± 4	118 ± 6

* Mean ± standard error.

protein fraction after Mepesulfate precipitation was only 22% of that present before precipitation (9 vs. 41 mg/100 ml). This finding confirms the observation made concerning cholesterol distribution in α- and β-lipoproteins of chick plasma. The chick, thus, differs from the human, but is similar to the dog in that a greater amount of cholesterol is bound to the α-lipoproteins than to the β-fraction. The results obtained from the ultracentrifugal analysis confirm the observation of Florsheim and Gonzales(5) concerning the completeness of Mepesulfate precipitation of β-lipoprotein from chicken plasma.

The amount of cholesterol bound to the α-lipoproteins was determined for chicks fed a 20% sesame oil meal semi-purified diet, the same diet with 2% added cholesterol or a stock diet. The results obtained are presented in Table I. The chicks fed the 20% protein diet or the stock diet had 72 and 73% of the total cholesterol bound to the α-lipoprotein fraction, respectively. Chicks in which the plasma cholesterol had been elevated by feeding cholesterol showed little change in the absolute amount of cholesterol bound to the α-lipoproteins; however, this accounted for only approximately 23% of the total plasma cholesterol. These data demonstrate that the cholesterol increase took place in the β-lipoprotein fraction. This is in agreement with observations in the human(14,15), but not with values reported for cholesterol-fed cockerels(13). Since the values reported by Orma(13) were determined on eluates of samples separated by paper electrophoresis, differences in methods may account for the variations noted.

In Table II are presented data on lipid distribution in plasma and α-lipoproteins for various species. Definite species differences in percentage distribution of the various lipid classes exist. The rabbit and guinea pig are similar to man in that less than 50% of the total cholesterol is bound to the α-lipoproteins. In the rat, dog, mouse and chick, however, more than 50% of the cholesterol is bound to the α-lipoproteins. A greater species variability is evident for lipid phosphorus and glyceride levels. In most species there is less lipid phosphorus bound to the α-lipoproteins than is the case in the human. The amount of glycerides bound to the α-lipoproteins is similar for man, guinea pig, dog and rat, with approximately 20% of the total glycerides found in the α-lipoprotein fraction. The rabbit has only 10% of the total plasma glycerides bound to the α-lipoproteins while for the mouse, 39%, and the chick

TABLE II. Lipid Distribution in Lipoproteins of Various Species Separated by Polyanion Precipitation.

Species	No. of animals	Cholesterol mg/100 ml Total	 α	Lipid phosphorus mg/100 ml Total	 α	Glycerides mM/L Total	 α
Man (male)	2	198	58	9.02	4.52	1.48	.28
Rabbit	4	40	9	3.33	.42	1.18	.12
Guinea pig	4	30	6	.68	.14	.43	.10
Rat	4	82	54	1.67	.84	.81	.17
Dog	5	186	156	11.49	4.33	.71	.15
Mouse	8	87	83	4.24	1.80	.49	.19
Chick	8	102	73	6.41	5.18	.41	.14

34% of the glycerides are found in the α-lipoprotein fraction.

The values reported demonstrate definite species differences in the percentage distribution of lipids bound to α- and β-lipoproteins. The method employed, because of its simplicity and accuracy, enables further studies of the role of the lipoproteins in lipid metabolism in both human and animal studies. The probable significance of the blood lipids and, consequently, lipoproteins in atherogenesis make such studies of considerable importance.

Summary. Paper electrophoresis and ultracentrifugation have been employed to demonstrate the completeness of β-lipoprotein isolation from chick plasma by a polyanion precipitation technique employing Mepesulfate as the precipitating agent. Data are presented demonstrating that in the chick made hypercholesterolemic by cholesterol feeding, the increase in plasma cholesterol occurs almost entirely in the β-lipoprotein fraction. Lipid distribution in the α-lipoprotein fraction of plasma has been determined for chick, rabbit, guinea pig, rat, mouse and dog as well as for the human. The chick, rat, mouse and dog were found to have more than 50% of the total plasma cholesterol bound to the α-lipoproteins in contrast to man, the rabbit and guinea pig. Values are also presented for distribution of lipid phosphorus and glycerides.

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Unilateral Renal Ischemia and Erythropoietin.[*] (26992)

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The presence of a circulating erythropoietic stimulating factor (erythropoietin) in the plasma of anemic animals has been demonstrated by a large number of investigators (1). Hypoxia is considered to be the fundamental stimulus for erythropoietin production and therefore erythropoiesis(2). The concept has been developed by Jacobson and his associates that rate of erythropoiesis is determined by the relationship between oxygen tension in the blood and oxygen demand by the tissues (including the site of formation of erythropoietin)(3). The failure of nephrectomized rats, but not ureter-ligated rats, to respond to a single massive hemorrhage or injection of cobalt with an elevation of plasma erythropoietin has led this group to

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[†] U.S.P.H.S. Postdoctoral Fellow.

conclude that this factor or a precursor is manufactured in the kidneys(4). This finding has been supported by several investigators(5-6). However, other workers have disputed the above conclusion(7-9).

If one assumes the validity of this hypothesis that rate of erythropoiesis is controlled by amount of circulating erythropoietin, and that its production (or that of a precursor) by the kidney is controlled by the oxygen supply-demand relationships of this organ, any procedure that would cause the kidney alone to be hypoxic, and still provide a normal oxygen supply to the rest of the body, should increase the production of erythropoietin and evoke a polycytosis in the animal. Unilateral partial ligation of the right renal artery has been used in these experiments to produce a renal stagnant hypoxia.

Methods. Male Sherman rats, weighing 375-475 g were anesthetized with a light dose of nembutal (1.8 mg/100g) plus ether (1st exp.) or ether alone (2nd exp.). The right kidney was exposed, using a lumbar approach. The right renal artery was constricted by a sterling silver clip that was compressed by a modified surgical needle holder. Degree of closure of the needle holder was controlled by an adjustable setscrew. This procedure, devised by Schaffenburg(10), offered a means of regulating precisely the amount of restriction of the arterial flow and therefore the degree of ischemia that would be obtained. A closure of the needle holder of 0.017″ (0.45 mm) measured with a mechanic's feeler gauge was used for the first series and a closure of 0.011-0.013″ (0.29-0.34 mm) for the second series. In the latter group, the smaller animals received the slightly tighter clamping of the clips.

The rats were exsanguinated under ether anesthesia by cardiac puncture approximately 24 hours after the ligation of the renal arteries. The blood was collected in heparinized syringes and centrifuged for 15 minutes at 3000 rpm. The plasma was frozen and stored until assayed for erythropoietic activity in the starved rat, using the Fe^{59} red blood cell incorporation method recommended by Keighley, *et al.*(11). The plasma from each donor was injected on 2 consecutive days into a single recipient animal, in order to detect any possible variations in the erythropoietic response of each of the artery-ligated rats. Plasma from identical, but unoperated rats was similarly collected and assayed.

Food was withheld from 16-20 young Sherman rats (male in Exp. 1, female in Exp. 2) at zero hours. Either 2 or 2½ ml of the plasma to be assayed was injected I.P. into these animals at 24 and 48 hours. One microcurie of iron-59 (specific activity 8.9 or 4.1 mc/mg) as ferric chloride diluted in ½ ml of 1% sodium citrate was given I.P. at 72 hours. The rats were sacrificed with ether at 90 hours. About 2 ml of cardiac blood was withdrawn into heparinized syringes, and ½ ml of this blood was pipetted into tubes for counting in a well type γ-ray scintillation counter (Baird Atomic model 810) and scaler (Nuclear model 163). Duplicate microcapillary hematocrit values were also obtained.

Results. The renal artery constriction produced by application of the silver clips with the modified needle holder having a 0.45 mm clearance between the jaws caused a moderate restriction of renal blood flow, but the blood vessel ligation using the 0.29-0.34 mm needle holder closure evoked a severe restriction of circulation to the kidney. Grossly, the more loosely ligated kidneys appeared at the time of sacrifice to be slightly enlarged, perhaps edematous, and somewhat pale or ischemic. The kidneys having the more severe restriction of blood flow showed a variable degree of enlargement, were also edematous, usually had an ischemic cortex, and had, in most instances, a hemorrhagic medulla.

Plasma taken from these rats failed on 2 separate occasions to induce a significant increase in the 18 hour uptake of radioiron into the red blood cells of the recipients (Table I) when compared with similarly treated controls that received plasma from unoperated animals in a standard and sensitive bioassay for erythropoietin. The "artery-ligated plasma" also failed to produce a change in hematocrit, the microcapillary hematocrit values being essentially the same for both experimental and control groups.

Discussion. Both a moderate and a severe restriction of renal blood flow was im-

TABLE I. Erythropoietic activity of plasma from rats having a constricted renal artery. Plasma was assayed by measuring RBC radioiron uptake and hematocrit in the recipient animals.

	Rats having constricted renal arteries and their controls (Plasma donors)				90 hr starved rats (Plasma recipients)				
Exp. no.	No. of animals per group	Body wt (g) Before	Body wt (g) After	Degree of Renal A. constriction (mm)	Body wt (g) Before	Body wt (g) After	Plasma dosage (ml × 2 days)	RBC Fe^{59} uptake* (mean ± S.E.)	Hct (%) (mean ± S.E.)
1.	10	443.2	424.3	.45	191.4	141.6	2.5	3.68 ±.28	51.0 ±1.0
	10	428.5	—	None	200.6	151.6	2.5	3.13 ±.23	49.2 ± .7
2.	8	452.8	431.9	.29-.34	140.0	109.5	2.0	2.74 ±.71	48.2 ± .6
	8	431.8	—	None	137.6	107.2	2.0	2.58 ±.56	46.8 ± .9

* Uptake in 18 hours expressed as % of injected dose.

posed in these 2 experiments since, on the one hand, there was evidence indicating that renal tubular function may be essential for erythropoietin secretion by the kidney(12), while, on the other hand, it was considered necessary to constrict the renal artery tightly enough so that a stagnant hypoxia sufficiently intense for stimulation of erythropoiesis might result. The restriction of arterial flow imposed in the first experiment caused a noticeable degree of renal ischemia, and, in the majority of another group of rats, a permanent renal hypertension (unpublished data). While the hemorrhagic renal medullae, observed in the majority of the kidneys ligated in the second experiment, implied that arterial pressure had been reduced to such a level that renal shutdown had occurred, evidence of shutdown was not seen in 2 cases, and in no instance was renal blood flow interrupted. There was no indication of a correlation between erythropoietic activity of the unpooled plasma from the artery-ligated rats, and the observed condition of the tightly ligated kidneys. Since the unoperated, contralateral kidney should have been able to compensate for the loss of its partially ligated partner, renal function should not have been impaired to any significant extent, and it was not expected that these animals would become uremic.

Naets(5), basing his idea upon the "cell separation theory" of Pappenheimer and Kinter(13) believed that the renal tubular epithelium was acutely sensitive to blood oxygenation and might respond to a lack of oxygen by increased erythropoietin production. The failure of unilateral partial ligation of the renal artery to cause an increase in amount of circulating erythropoietin suggests that Naets' inference may not be correct and that the control over production of this factor may be exercised by some other organ. Seip (14) and Osnes(15) have suggested the hypothalamus as the site of this regulation, through either a nervous or neural humoral mechanism.

Recently, Kuratowska(16) has reported that the perfusion of rabbit kidneys with hypoxic blood induced the production of erythropoietin. A reticulocytosis and an increase in percentage of marrow erythroblasts was evoked in mice receiving boiled filtrates of the perfusate. Administration of filtrates of the fully oxygenated blood perfused through the rabbit kidneys caused only a slight reticulocytosis and no increase in marrow erythroblasts in the test animals. Nonperfused rabbit blood was also assayed. This work has been confirmed by Fisher and Birdwell(17), who perfused dog kidney-lung preparations with blood containing cobalt. The dialyzed plasma was assayed by measuring Fe^{59} RBC incorporation in starved rats. The possibility exists that the differences in response were due to the use of perfused kidneys by the former investigators and intact animals by ourselves.

The other possible explanations for this negative result are that partial ligation of an artery may be an inadequate means of inducing renal hypoxia, or that insufficient quantities of erythropoietin are engendered by unilateral renal hypoxia over a 24 hour period. A moderately severe reduction in renal artery pressure may, by an interpretation of the "cell separation theory," cause an increase in the cell percentage of the normally cell-poor fraction of blood circulating through the peritubular epithelium, and the tissue would therefore receive an adequate supply of oxygen. A more severe and uncompensated restriction in arterial flow might result in failure of tubular function and inability to secret erythropoietin. However, there is evidence (unpublished data) that the juxaglomerular apparatus secreted renin in a group of rats having chronically and severely ligated kidneys. An extended rise in blood pressure was noted in these animals. Since ischemia was seen in even the series of moderately restricted kidneys, the tubular epithelium may very likely have been hypoxic.

Summary. Plasma taken from rats having moderately constricted or severely constricted right renal arteries failed on 2 occasions to induce a significant increase in the 18 hour RBC uptake of Fe^{59} when compared with similarly treated controls that received plasma from unoperated animals. Microcapillary hematocrit values were also the same for the experimental and control assay groups.

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Effect of Ribonucleic Acid Extracts upon Viability of Skin Homografts in the Rat.*† (26993)

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The phenomenon of "actively acquired tolerance" has been described as an induced state in which tissue homografts become immunologically acceptable to hosts which normally would react against them(1). In these now classic experiments, tolerance of skin grafts was produced by exposure of the hosts to foreign homologous tissue cells sufficiently early in foetal life. Further studies have demonstrated the induction of actively acquired tolerance of homologous tissues in newborn mice(2) and rats(3). An extensive series of investigations by the Minnesota group has provided ample evidence that in-

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† Abbreviations: EDTA, ethylenediaminetetraacetate; Tris, tris (hydroxymethyl) aminomethane; ATP, adenosine triphosphate.

duction of tolerance may also be observed in more mature animals(4-6).

In the above reports tolerance was produced by administration of viable cells or through parabiotic union. Attempts to produce tolerance with cellular extracts have generally proven unsuccessful(7-9). Accordingly, the recent study of Ashley *et al.* (10) in which tolerance of skin grafts was induced in neonatal rats by administration of ribonucleic acid (RNA) extracts was of considerable interest. In the present study, we have attempted with various modifications to reproduce these results with olders rats.

Methods and Materials. Animals and diets. Male, weanling rats of the Long-Evans strain and male rats of the Holtzman strain, weighing 180-200 g, purchased from commercial sources, were housed in individual cages. The Long-Evans animals, serving as skin recipients, were fed a purified, basal diet and daily vitamin pills described previously(11). Holtzman animals, serving as skin and RNA donors, were fed a commercial stock ration (Purina chow). All diets were consumed *ad libitum.* Solutions of deoxypyridoxine (25 μg/ml) and pyridoxine (1 mg/ml) were prepared in isotonic sodium chloride and adjusted to pH 7.0 for intraperitoneal injection.

Grafting procedure. Pieces of skin of full thickness, measuring 2.5 × 3.5 cm, were excised from the anterior abdominal wall of the donor animal and applied to the host area as described previously(12). In the present experiments, the recipient bed was prepared by removing a similar-size piece of skin from the mid-dorsum as described by Hansen and Hubay(13). Pressure dressings were applied for one week subsequent to grafting. A protective dressing consisting of a Telfa pad held in position by narrow strips of adhesive was maintained during the remainder of the experiment. The criteria utilized to judge survival of a skin graft have been presented elsewhere(12,14).

Preparation of RNA extracts. Approximately 85% of the spleen was removed from prospective donor rats, frozen on solid carbon dioxide, and stored at —5°C until extraction one week later. RNA was extracted from the various cellular fractions described below by the phenol method of Gierer and Schramm(15) and residual ether removed from the final extracts by bubbling with nitrogen. All extracts were adjusted to pH 7.0 prior to injection.

1) *Spleen homogenate.* A 10% (w/v) homogenate of spleen in a solution of 0.01 M sodium citrate and 0.14 M sodium chloride of pH 7.0 was prepared from minced spleen with a hand homogenizer.

2) *Cytoplasmic Fraction.* This fraction was prepared by the method of Colter and Brown (16).

3) *Mitochondrial, Microsomal, and Soluble Fractions.* These fractions were obtained by differential centrifugation at 0°C of a splenic homogenate prepared as described under Section 1 above. The method utilized in the present study incorporated various features of procedures employed for isolation of liver subcellular fractions(17-19) and was conducted as follows. The supernatant resulting from centrifugation of the splenic homogenate at 2000 × g for 10 minutes was centrifuged at 6590 × g for 20 minutes to yield a mitochondrial precipitate and a supernatant fraction. Centrifugation of the latter at 105,000 × g for 80 minutes precipitated a microsomal pellet. The remaining supernatant constituted the soluble fraction.

Analytical procedures. 1) RNA was determined by the orcinol reaction(20) and protein by the method of Lowry *et al.*(21). 2) For electron microscopy, the microsomal pellet was fixed in buffered osmium tetroxide, dehydrated and embedded in Vestopol W. Sections displaying gold interference colors were examined in a Philips 200 electron microscope. 3) *Cholinesterase assay.* Cholinesterase activities of a splenic homogenate, mitochondrial and microsomal fractions of spleen were determined by a null point potentiometric titration procedure conducted with a pH-stat.‡ The splenic homogenate was prepared by homogenizing one gram of spleen in 10 ml of a solution of 0.01 M sodium citrate and 0.14 M sodium chloride of pH 7.0. Total mitochondrial and microsomal pellets prepared as described above by differential centrifugation of the homogenate were homogenized in 10 ml of a pH 7.4 buffer

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‡ TTTI b Titrator Radiometer, Cophenhagen.

TABLE I. Analytical Data of Various Splenic Fractions.[1]

Fraction	Mg RNA/ 100 mg spleen[2]	Mg RNA/mg fraction protein	Specific cholinesterase activity[5]
Original Homogenate	.55	.033	.017
Cytoplasmic[3]	.38	.058	
Microsomal[4]	.17	.17	.22
Mitochondrial[4]	.05	.02	.038
Soluble[4]	.35	.05	

[1] RNA was determined in extracts prepared from the various fractions by the phenol method.

[2] Fresh weight.

[3] Isolated by method of Colter and Brown(16).

[4] Isolated by differential centrifugation of original homogenate.

[5] Expressed as μmoles of substrate hydrolyzed per min. per mg of protein.

consisting of 0.1 M tris, 0.001 M EDTA and 0.001 M ATP. The final reaction volume (4.0 ml) consisted of 0.2 ml of the mitochondrial or microsomal homogenate, 0.2 ml of 0.25 M $MgCl_2$, 0.4 ml of 0.03 M acetylcholine iodide and 3.2 ml of ion-exchanged distilled water. An aliquot (0.2 ml) of the original homogenate was assayed in an identical fashion with the exception that 0.2 ml of water was replaced by an equal volume of the tris buffer. Titrations were performed with 0.30 N NaOH at 30°C and pH 7.4.

Experiments and results. Analytical data obtained for the various splenic fractions are presented in Table I. Determinations of both cholinesterase activity and RNA content proved useful in assessing purity of the mitochondrial and microsomal fractions. The validity of the cholinesterase measurements as a microsomal marker derives from observations that this enzyme is concentrated in the microsomal fraction of brain(22,23) and muscle.§ With the assumption that mitochondria are free of RNA and cholinesterase, one can calculate that this fraction prepared by our procedure is contaminated with either 11% (RNA data) or 17% (cholinesterase data) of microsomes. Thin sections of the microsomal pellet examined in the electron microscope consisted of granules approximately 150Å in diameter and some rough-surfaced membranes characteristic of a microsomal fraction. No mitochondria were observed in sections of the microsomal pellet.

Fig. I depicts growth responses of Long-Evans recipient rats. Following a 2-week period during which weanling rats consumed a diet furnishing 10 μg of pyridoxine daily, the animals were fed a pyridoxine-free diet and given daily intraperitoneal injections of 25 μg of the pyridoxine antagonist, deoxypyridoxine, per 100 g of body weight for 3 weeks. During this time, growth ceased and skin symptoms characteristic of pyridoxine deficiency appeared. After 2 weeks of pyridoxine deprivation, each experimental animal received a single, intraperitoneal injection of RNA extracted from the total cellular fraction (Table II) derived from the spleen of its prospective skin donor. Each RNA extract was obtained from a separate spleen. One week following RNA administration, deoxypyridoxine treatment was discontinued and the animals transferred to a dietary regimen furnishing 50 μg of pyridoxine daily and given 3 daily intraperitoneal injections of one mg of pyridoxine per 100 g of body weight. After one week of pyridoxine therapy which produced immediate growth responses and gradual alleviation of the skin symptoms, the Long-Evans animals received skin homografts from Holtzman rats. Thus, one Holtzman rat donated both skin and RNA extract to a single Long-Evans recipient. For the remainder of the experiment,

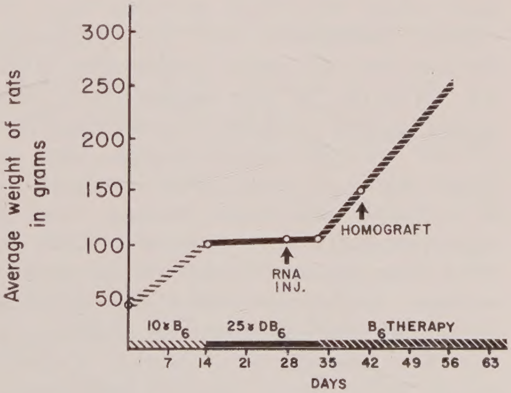

FIG. 1. Growth responses of Long-Evans recipient rats. DB_6 refers to deoxypyridoxine.

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§ Unpublished studies of Drs. J. Smith and R. Basford of these laboratories have demonstrated that rat brain and muscle mitochondria contain 5% of microsomes as determined by cholinesterase assay.

TABLE II. Effect of RNA on Viability of Skin Homografts.[1]

				% homograft survival (weeks after grafting)					
Source of splenic RNA	No. of recipients	No. of exp.	RNA (μg)/100 g body wt[2]	2	3	4	6	8	12
(Controls)	175	17	0	0					
Original Homogenate	40	4	1300-1800	30	0				
Cytoplasmic Fraction	20	2	1170-1500	30	0				
Soluble "	20	2	1250-1580	0					
Microsomal "	10		455-590	100	60	20	0		
	8		327-490	100	100	87	37	0	
	10		750-792	50	0				
	10		490-580	100	100	100	80	40	10[3]
	10		400-450	100	100	70	20	10	10[3]
	10		490-580	100	100	60	30	0	
	12		490-580	100	100	100	25	8	8[3]
	10		490-580	100	100	100	50	10	10[3]
	15[3]		490-580	100	100	—			

[1] Results of individual experiments with microsomal RNA are presented. Composite data are given for those groups receiving RNA derived from other fractions and for control groups.

[2] Range of values is presented.

[3] Animals still under observation.

the recipient animals received the purified diet furnishing 50 μg of pyridoxine daily. Except for omission of RNA, treatment of the control Long-Evans recipients was identical with that described above. Equal numbers of control and experimental recipients were utilized in every experiment.

The effect of RNA extracts upon the viability of skin homografts is shown in Table II. In controls, initial signs of rejection were evident 10 days after grafting and in 175 animals the skin homografts were completely rejected within 2 weeks. In no case did a control homograft survive beyond this period. Thirty per cent of the skin homografts in animals treated wih RNA extracted from the original homogenate and the cytoplasmic fraction were still viable 2 weeks after grafting. However, all skin homografts in these groups, as well as in the one receiving RNA derived from the soluble fraction, were rejected within 3 weeks after homografting. In 8 separate experiments, however, 57 of 80 skin homografts in rats receiving RNA extracts from the microsomal fraction were in excellent condition 4 weeks after grafting. All grafts in the last experiment, still in progress, are surviving at 3 weeks. Unfortunately, the inordinate degree of graft mutilation beyond the 4-week period makes it difficult to assess the effects of RNA upon further prolongation of the skin homografts. In some experiments as high as 70% of the grafts surviving at 4 weeks were lost in this manner. We are at a loss to account for this high degree of graft mutilation which has not been observed over a 4-year period during which homotransplantation experiments have been conducted in our laboratory. Our extensive experience with skin homografts(12, 14) would lead us to expect a high survival rate in grafts which are in such excellent condition at 4 weeks. The percentage survival figures (Table II) are based upon the number of original grafts in each experiment, and, accordingly, present a conservative estimate of the RNA effect.

Discussion. Data presented here have indicated that the viability of skin homografts can be enhanced if the recipient is pre-treated with microsomal RNA extracts while in a state of pyridoxine deficiency. This beneficial effect of RNA agrees with the findings of Ashley *et al.*(10) in neonatal rats. The inefficiency of large doses of RNA derived from the original splenic homogenate or the cytoplasmic fraction has no ready interpretation. The presence of molecular species of RNA in these fractions which are antagonistic to microsomal RNA may afford a possible explanation. There is also some basis for the belief that the dosage of RNA may be a critical factor. This possibility gains credence from the observation that, in one experiment (Table II), higher doses of microsomal RNA were ineffective. This question can only be resolved by further experimentation with varying doses of microsomal RNA administered singly or in combination with RNA derived from other fractions. Clarification of the specificity of RNA will derive from studies in which RNA of the extracts is selectively removed by the action of ribonuclease.

The role of microsomal ribonucleoproteins in protein synthesis has been documented (24) and a variety of experiments have indicated that administration of RNA or ribonucleoprotein may lead to *in vivo* protein synthesis(25-28). It may, therefore, be presumed that administration of microsomal RNA in the present study leads to the formation of donor antigens in the host. For induction of actively acquired tolerance, antigenic stimuli are generally applied to the host while it is in a state of immunological unresponsiveness. For this purpose, various experimentors have utilized the foetal or newborn animal(1-3) as well as older recipients treated with cortisone(29), x-irradiation(30) or 6-mercaptopurine(31). We administered RNA during a period of pyridoxine deficiency since previous studies have shown that this deficiency can produce a desired state of immunological inertness(32). The experimental approach of the present study was, therefore, designed to simulate the more classical methodology utilized for induction of acquired tolerance. On the basis of his nucleation theory of antibody production, Goldstein(33) has suggested a mechanism for production of acquired tolerance by the use of antimetabolites. Our findings could be interpreted within the framework of this rationale.

Summary. Administration of an RNA extract prepared from the microsomal fraction of splenic cells increased the viability of skin homografts. RNA derived from a normal, adult rat which later served as the skin donor was administered to a pyridoxine-deficient rat of another strain. Skin homografting was performed after the RNA-treated recipient had received intensive therapy with pyridoxine.

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